UNIVERSITY OF ALBERTA

THREONINE REQUIREMENT AND THE EFFECT OF THREONINE ON GUT MUCIN CHARACTERISTICS IN PIGLETS RECEIVING INTRAGASTRIC NUTRITION

by

GARSON LAW

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ABSTRACT

Threonine (THR) is the second limiting amino acid in many grain based pig diets and is involved in the structure and function of intestinal mucins. THR requirement was determined in piglets (n=26, 10 ± 0.5 d old, 2.5 ± 0.5 kg) receiving an elemental diet intragastrically (IG). The THR requirement was 0.42 g/kg/d, vs. other oral estimates (0.6 g/kg/d) and to an identical intravenous (IV) feeding study (0.19 g/kg/d). The difference was hypothesized to be due to THR use in gut mucin production. Piglets (n=21, 2± 0.5 d old, 1.8± 0.3 kg) were fed one of three THR diet treatments for 8 days: 1) adequate (0.6 g/kg/d IG), 2) deficient (0.1 g/kg/d IG), or 3) supplemented (0.1 g/kg/d IG + 0.5 g/kg/d IV). THR-deficient piglets had lower mucosal weight and deposited less protein. Histochemical and mucin analysis showed greater gut mucin content in THR-adequate piglets. Although THR supplied IV was partially available for use, orally-supplied THR was preferred.

DEDICATION

To my parents

To my best girl

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1. INTRODUCTION

Premature birth is a leading cause of human infant morbidity and mortality. Low birth weight (LBW) infants often have an inability to tolerate oral feedings due to a variety of factors, including short bowel syndrome, gastrointestinal surgery, chronic severe diarrhea, immature bowel function (Hay, 1986), and respiratory diseases (Adamkin, 1986). In these instances, the provision of nutrition by parenteral means is critical. However, the levels of amino acids administered must be high enough to meet the demands for protein synthesis and not in excess to the point of creating stress on immature enzymatic metabolic systems, resulting in possible toxicity. Due to ethical and practical constraints, the neonatal piglet is often used as a model to study nutritional metabolism in the human neonate because of the many similarities in anatomy, physiology, metabolism and nutrition between the two species (Moughan and Rowan, 1989).

Threonine (THR) is an indispensable amino acid that is found in high concentrations in a number of gastrointestinal (gut) secretions that act to protect the mucosa from digestive proteases, prevent the dehydration of the underlying mucus membranes and protect the gut wall from microorganisms and parasitic invasion (Toribara et al. 1993). Thus, threonine is believed to play an important role in the proper development of the neonatal gut. The threonine requirement for piglets of approximately 8 days in age has been determined by Chen (1997, thesis) to be 0.20 g/kg/d when fed intravenously, while the oral requirement has been estimated at 0.61 g/kg/d (NRC 1998), a 3-fold difference. In similar studies involving lysine requirement of piglets receiving total parenteral nutrition (TPN), the requirement was observed to be similar (0.79 g/kg/d, House et al. 1998) to

published estimates (0.86 g/kg/d, NRC 1998). This suggests that the gut either metabolizes a significant amount of threonine for its own use or is responsible for threonine losses in the body. Stoll et al. (1998) have suggested that in piglets 60% of dietary threonine is utilized by the gut, compared to lysine (35%), leucine (32%), and phenylalanine (35%). Furthermore, they showed that nearly 90% of this metabolized threonine was either secreted as mucosal protein or catabolized. It follows then that since gut atrophy is known to occur as a consequence of parenteral feeding, the dietary requirement of threonine would be also be diminished. The difference observed in the apparent needs of dietary threonine between oral and intravenous feeding need to be confirmed empirically, however, since published oral estimates of threonine requirement are based upon reference proteins. If a true difference exists, then the cause of the difference needs to be further examined.

The literature review will discuss threonine metabolism and requirements, and will review the role of threonine in the gut in order to determine which direction further research should take in investigating the difference between oral and intravenous requirement of threonine.

2. LITERATURE REVIEW

2.1 THREONINE METABOLISM

L-threonine is unique due to the existence of two specific degradative pathways. These two major pathways catalyzing the degradation of L-threonine in the liver of mammals are 1) threonine dehydratase (EC 4.2.1.16; TDH), and 2) L-threonine 3-dehydrogenase (EC 1.1.1.103; TDG). **(Fig. 2.1.1)**

Catabolism by TDH in rat hepatic mitochondria yield as final products NH_4^+ and 2-ketobutyrate (KB) (Freedland et al. 1964), which may then be rapidly converted to either proprionic acid and CO_2 by pyruvate dehydrogenase (branched-chain α-ketoacid dehydrogenase) or 2-aminobutyric acid via aminotransferase (Steele et al. 1984). Proprionyl CoA can be converted to succinyl CoA, leading to gluconeogenesis via the citric acid cycle. Thus, TDH serves to mobilize threonine carbons for hepatic gluconeogenesis during starvation or in response to a high protein diet (Mak et al. 1981). TDH is inducible with changes in feeding and is believed to be involved in the homeostatic regulation of free threonine concentrations (Ishikawa et al. 1975). While TDH is involved in the gluconeogenic pathway in rats it represents only 1-3% of TDG activity in pigs (Le Floc'h et al. 1995).

Catabolism by rat hepatic TDG in the fed state yields 2-amino-3-ketobutyrate (ABA), which is then mainly cleaved by 2-amino-3-ketobutyrate CoA ligase to acetyl-CoA and glycine (Bird et al. 1984; Dale 1978). About 10% of ABA may spontaneously decarboxylate to form aminoacetone via 2-amino-3-ketobutyrate CoA ligase (Bird and Nunn 1983). At low levels of L-threonine, the rate of glycine production is several times

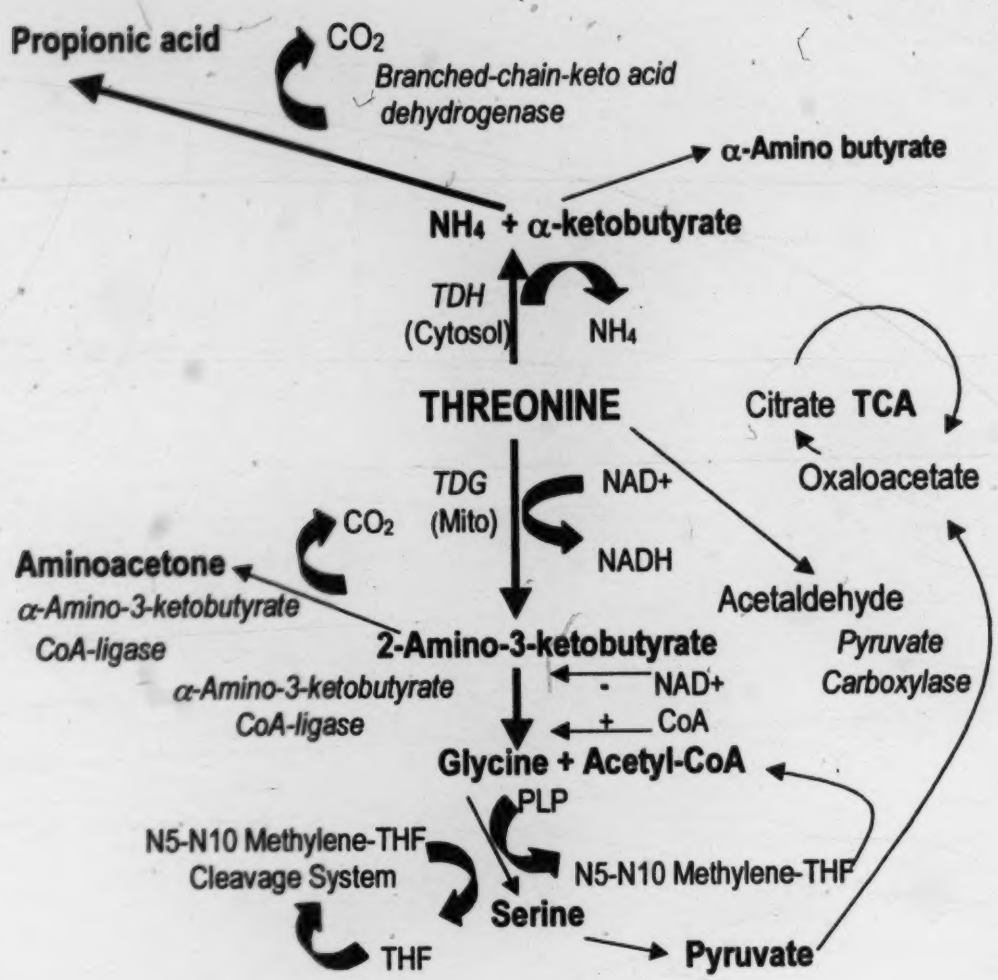


Figure 2.1.1: Pathway of threonine metabolism in mammals.

greater than the rate of aminoacetone formation. Glycine may either be oxidized by the mitochondrial glycine cleavage system, or incorporated into other cellular constituents (Neuberger, 1981). Acetyl CoA may either by oxidized via the tricarboxylate cycle, or transported from mitochondria by way of citrate for lipid synthesis. TDG activity seems to be dominant during the normally-fed state when the requirement for hepatic gluconeogenesis is low. In this state, TDG is likely responsible for maintaining homeostasis of free somatic threonine concentrations derived from the diet by directing excess threonine carbons to oxidative or biosynthetic processes (Bird and Nunn 1983).

Metabolism by a third enzyme, threonine aldolase produces acetaldehyde and glycine, although the activity of threonine aldolase has been found to be low in rat liver, regardless of the metabolic state of the animal (Bird et al. 1984, Bird and Nunn 1983).

2.2 THREONINE REQUIREMENT IN DIFFERENT SPECIES

The specific oral requirements of threonine in neonatal piglets have seldom been emipirically determined. Estimates have usually been calculated based upon data from older piglets and are compared to the threonine content of various standards (**Table 2.2.1**). The threonine requirement in piglets weighing 1-5 kg has been estimated in this manner to be 3.4 g/100 g protein, or about 0.6 g/kg body wt/d (NRC 1998). This calculation is extrapolated from various studies that have determined the threonine requirement in piglets to be 9.2 g/kg diet, or 3.68 g/100 g of crude protein (Sewell 1953); 6.07 g/kg diet, or 2.53 g/100 g of crude protein (Kim et al. 1983); and 6.6 g/kg diet, or 2.97 g/100 g of crude protein (Leibholz 1988). The threonine content in piglet tissue at 7

days of age was found to be comparable at 3.9 g/100g protein while the content in swine milk, human milk, and egg protein was 3.7, 4.4, and 5.3 g/100g protein, respectively (Aumaitre and Duee 1974, Ciba Giegy 1970, Davis et al. 1994).

Table 2.2.1: Threonine requirements in pigs & humans compared to concentrations in reference proteins

	Piglet Req.[1]	Human Req.[2]	Piglet Tissue[3]	Human Fetal Tissue[4]	Swine Milk[5]	Human Milk[5]	Egg Protein[6]
THR (g/100g protein)	3.4	4.0	3.9	4.1	3.7	4.4	5.3

[1] Calculated from NRC 1998, for swine weighing 1 to 5 kg.
[2] Calculated from FAO/WHO 1990, for infants 3 to 4 months of age.
[3] From Aumaitre and Duee 1973, in tissue of piglet at 7 days of age.
[4] Calculated from Widdowson 1979, in tissue of fetuses 2.9 to 3.4 kg.
[5] From Davis et al. 1994
[6] From Ciba Giegy 1970

In human infants 3-4 months of age, the threonine requirement was estimated to be 4.0 g/100 g protein and similar to its concentration in human fetal tissue (FAO/WHO 1990, Widdowson 1979). The similarity between these estimates and that for piglets lends support for the use of piglets as an experimental model in investigating threonine requirements in human infants.

In piglets fed a completely elemental diet by total parenteral nutrition (TPN), the threonine requirement was determined to be 0.2 g/kg/d, or about 13.5 mg/g amino acids (Chen 1997). This suggests that a 3-fold increase in the need for threonine exists when piglets are fed orally vs intragastrically. This comparison must be viewed carefully however as the oral estimates are based upon semipurified protein diets while the

intravenous data comes from piglets fed an elemental diet consisting of amino acids without protein. Furthermore, there is a difference in the route of administration of the respective diets and any difference in threonine requirement may be due to either first-pass metabolism or by some other physiological phenomena. For example, gut atrophy is known to occur in piglets fed by TPN (Goldstein et al. 1985) and since threonine is an important constituent of intestinal mucus, the higher threonine requirement in orally-fed piglets may be due to a higher demand for threonine by the gut.

In orally-fed kittens of approximately 13 weeks of age (1.4 kg), the threonine requirement was found to be 5.0 g/kg of diet comprising of 150 and 200 g crude protein/kg diet. When crude protein was 300 or 500 g/kg diet, the requirement of threonine increased to 6.0 g/kg diet. This amounted to about 2.5 and 1.2 g/100 g protein, respectively (Hammer et al. 1996). In 1-day old broiler chicks (157 g) fed a maize-soyabean-meal diet consisting of 230 g protein/kg diet, the maintenance requirement for threonine was found to be 45.7 mg/kg body wt/d, which represented 5.5 % of the total threonine requirement of about 0.83 g/kg body wt/d (4.1 g/100 g protein) for young chicks (Edwards III et al. 1997, NRC 1994).

The dietary threonine requirement for several species and the ratio of threonine to protein is consistently in the range of about 3.5 to 4.5 g/100 g protein, implying that the optimal intake of threonine lies within this range The notable exception is the kitten, where this ratio was found to be about 2.5 g/100 g protein. This suggests that the requirement for threonine may be lower in carnivores than in omnivores, owing perhaps to a difference in digestive function and gut metabolism.

2.3 THE ROLE OF MUCUS IN THE GASTROINTESTINAL TRACT

Threonine is an important constituent of the mucus layer lining the gastrointestinal tract, specifically in mucin proteins (Lamont 1992, Specian and Oliver 1991). Therefore, the need for threonine in mucin synthesis may help explain the large proportion of dietary threonine requirement used by the gut (Chen 1997, Stoll 1998). If a difference in threonine requirement between intravenously and intragastrically fed piglets is confirmed, this would imply that a great deal of threonine is absorbed and utilized by the gut. Within the gut, THR is found in high concentration in a number of gastrointestinal (gut) secretions that act to protect the mucosa from digestive proteases, prevent the dehydration of the underlying mucus membranes and protect the gut wall from microorganisms and parasitic invasion (Toribara et al. 1993). In particular, THR (along with serine and proline) is highly concentrated in the mucin proteins of intestinal mucus secretions (**Table 2.3.0.1**).

Table 2.3.0.1 Amino Acid Composition of Crude Mucin from Intravenously-Infused Pigs

Amino Acid	% Crude Mucin	SEM (n=3)
Arg	2.4	0.03
His	1.3	0.07
Ile	2.7	0.07
Leu	5.2	0.3
Lys	2.3	0.14
Met	0.6	0.07
Phe	2.5	0.16
Thr	16.4	0.33
Val	6.0	0.06
Ala	9.9	0.63
Asp	7.0	0.34
Glu	8.2	0.29
Gly	8.7	0.65
Pro	12.4	0.69
Ser	12.4	0.74
Tyr	2.1	0.13

Adapted from Lien et al. 1997

Because gut atrophy is a commonly associated drawback of parenteral nutrition, oral intake of threonine may play an important role in the luminal support of proper neonatal gut development.The following sections will review various aspects of the structure and function of gastrointestinal mucins.

2.3.1 FUNCTIONS OF MUCUS

The mucus gels of the gastrointestinal tract are comprised of water (95% /w) and organic materials such as lipids, proteins, ions, and enzymes that are likely acquired from epithelial secretions and from exfoliated and disrupted cells (Neutra and Forstner 1987).

The gels cover the entire luminal surface of the gastrointestinal tract and its primary function is to protect the underlying epithelium (Neutra and Forstner 1987; Lamont 1992). In conjunction with bicarbonate, mucus protects the epithelium from digestive processes and corrosive gastric juices by creating an unstirred layer and by acting as a diffusion barrier, preventing large molecular weight compounds such as proteolytic enzymes from reaching the epithelium. Mucus also traps toxins and bacteria to prevent infection. In the intestinal lumen, adherent and soluble mucus act as a lubricant in providing protection from mechanical damage caused by the passage of food. Mucus also serves an important function in the digestive process by creating a digestive zone in which digestive enzymes are immobilized near the epithelial surface. This prevents their removal by peristalsis and places them in a favourable position for the digestion and absorption of substrates (Toribara et al. 1993).

2.3.2 STRUCTURE OF MUCINS

One of the major components of mucus gels are large molecular weight glycoprotein monomers (2×10^6 daltons) known as mucins (Allen 1981). The monomers are comprised of four subunits (5×10^5 daltons) that are linked by disulphide bonds and arranged into the three-dimensional polymeric structure necessary for gel formation since glycoprotein subunits reduced by mercaptoethanol or proteolysis lack the ability to form gels (Allen et al. 1984) (**Fig. 2.3.2.1**). Two different models have been proposed for the polymeric structure of mucin which tends to exist in a flexible spheroidal shape in solution. Allen (1981) proposed a four bladed windmill structure in which the protein

backbones of all four mucin subunits are linked in a common area known as the non-glycosylated 'naked' region. Carlstedt and Sheehan (1984) proposed that mucin was shaped as a long peptide containing several glycosylated and non-glycosylated regions, resembling a coiled thread. Mucin polymers overlap and are noncovalently joined to provide the structural basis of the gel. These noncovalent interactions are resistant to osmotic pressure and solubilization but not against gel spreading or mechanical disruption (Bell et al. 1985). The structure-function relationships of intestinal mucins are summarized in the **Table 2.3.2.1**:

Table 2.3.2.1 Structure-Function Relationships of Intestinal Mucins

Structural Feature	Property of Function
Very high molecular weight	enhances polymerization
Extended rod-like structure	high viscosity
Glycosylated repeating sequences	protease resistance
Branched oligosaccharides	binding of toxins, bacteria
Hydrophobic domains	lipid binding

from Lamont (1992).

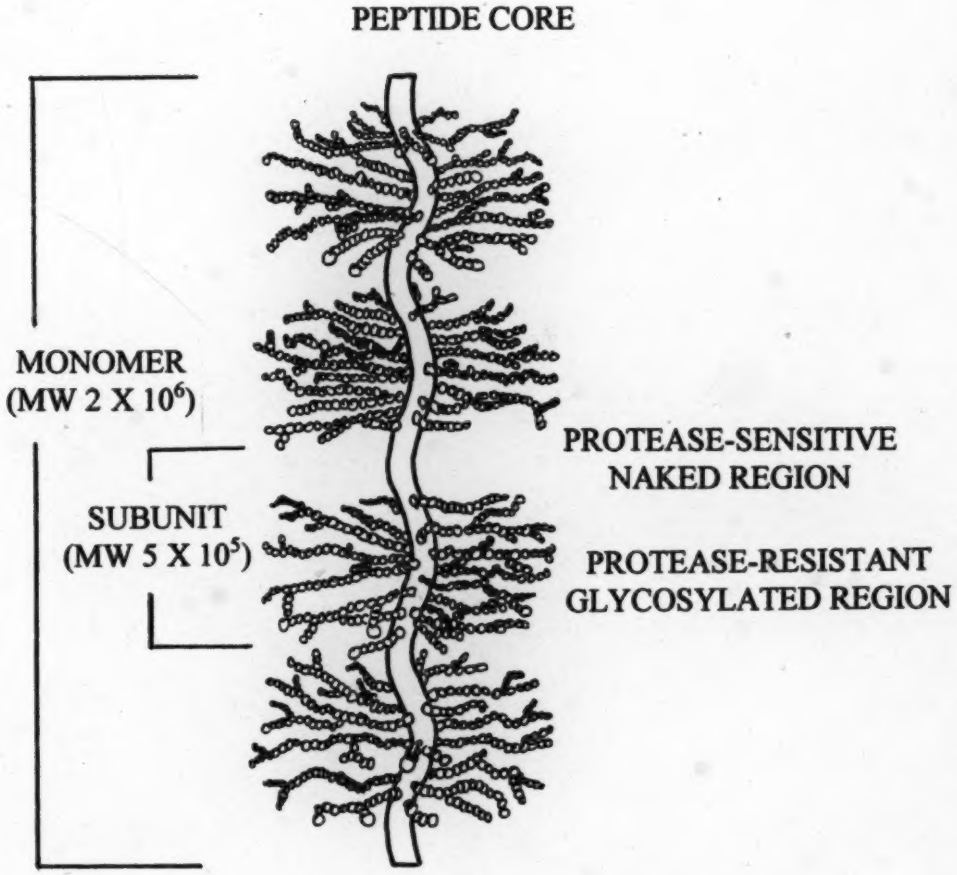


Figure 2.3.2.1: "Bottle-brush" structure of intestinal mucin protein (adapted from Lamont 1992).

There are two major protein regions in mucin. The glycosylated (native) region accounts for over 95% of the glycoprotein and about 65% of the total protein in mucin. Colonic mucins are less glycosylated than small intestinal mucins, comprising 62% of mucin by weight in rat colon and 67-80% in human colon (Specian and Oliver 1991). The protein in this region is rich in proline, serine, and threonine. Serine and threonine residues along the protein core provide the attachment sites for the oligosaccharide sidechains while the high proline content may act to maintain the shape of the protein core, allowing the carbohydrate chains to be packed closely together, resulting in resistance to proteolytic attack (Forstner and Forstner 1986). Proline is also believed to prohibit α-helix formation and preventing compact folding of the polypeptide, thus maintaining an expanded conformation that facilitates a high degree of glycosylation (Allen 1978). Serine and threonine comprise 25% of total amino acid residues in rat colonic mucins, 57% of rat small intestinal mucins and 41% of human small intestinal mucins while proline may account for up to 15% of the total amino acid residues (Specian and Oliver 1991). This region is characterized by repeating units of 15-30 amino acids which form the basic structural unit of the glycosylated region and allow the goblet cell to assemble a very long glycoprotein by assembling 20 or more of these repeats in an end-to-tail fashion (Lamont 1992).

The non-gycolsylated (naked) region represents about 35% of the total protein and about 4-5% of the total glycoprotein. This region has an amino acid composition similar to that of the average globular protein, rich in glutamate and aspartate and unique in that

it is rich in cysteine. This is consistent with its role in the formation of the polymeric structure of mucus via the joining of mucin subunits by disulphide bridges (Allen 1978, Allen 1981, Lamont 1992). Because this region lacks the protection of closely packed oligosaccharide sidechains, it is pronase sensitive. The globular peptide also has considerable hydrophobic regions which may be involved in lipid binding and also in protein-protein interactions (Lamont 1992).

The mucin protein itself consists of a peptide backbone surrounded by oligosaccharide chains, forming a 'bottle-brush' structure (Allen 1981) (**Fig. 2.3.2.1**). Regional differences in the challenges faced by gastrointestinal mucins are reflected in the heterogeneity of their compositions. There is also a large degree of microheterogeneity in mucin composition of the same region of the gastrointestinal tract. Still, there exists several common features. Mucins are characterized by a high carbohydrate content (>80% by dry weight) and a relatively low protein content (15-20%), with sulphate at 2-7%. The carbohydrate contents consist of five different monosaccharides: galactose, fucose, N-acetylgalactosamine, N-acetylglucosamine, and sialic acid (Allen 1978, Specian and Oliver 1991). Uronic acids (common in connective tissue proteoglycans), mannose (common in serum and epithelial glycoproteins), and glucose are not found in mucin glycoproteins.

Mucin carbohydrates are arranged in linear or branched oligosaccharide chains varying in length from 2-22 sugars and are always O-glycosidically linked via N-acetylgalactosamine to the hydroxyl group of either serine or threonine in the protein core. (**Fig. 2.3.2.2**) Chain elongation occurs by alternating the addition of N-

acetylglucosamine and galactose. Chain elongation begins with N-acetylglucosamine in the small intestine while beginning with N-acetylglucosamine in gastric mucin. Branches develop via glycosidic bonding of N-acetylglucosamine to either galactose or core N-acetylgalactosamine.

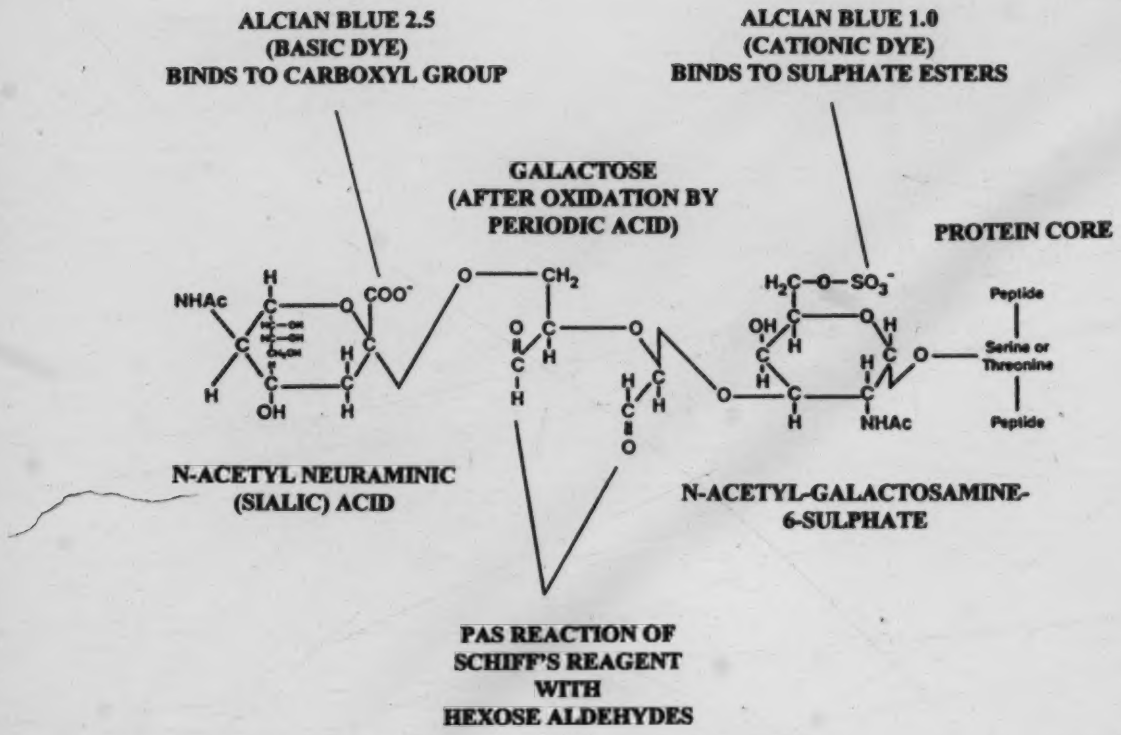


Figure 2.3.2.2: Oligosaccharide attachment to mucin protein core and sites of histochemical stain binding.
(adapted from Spicer and Schulte 1992).

2.3.3 SYNTHESIS OF MUCIN

Mucin is synthesized in intestinal epithelial goblet cells by the rough endoplasmic reticulum (RER) and passed to the Golgi apparatus, where it is combined with carbohydrates and packaged into membrane bound, secretory vacuoles called mucigen granules. The mucigen granules aggregate in the distended apical cytoplasm, are released by exocytosis, and combine with water to form mucus (Wheater et al. 1987). Goblet cells, which are more prevalent in the lower than in the upper small intestine, are derived from the undifferentiated cells lining the intestinal crypts. The undifferentiated cells are the most abundant cell type in the crypts and are extremely proliferative. Because of their high mitotic rate, they are very sensitive to radiation, ischemia, and antimitotic agents such as methotrexate and colchicine. As they divide, the cells migrate up the crypt epithelium and differentiate into the mature specialized cells that populate the villi. Cells differentiating into goblet cells continue to migrate up the villus until they arrive at the tip, from which they are eventually extruded into the lumen. Normally, the villus cell population is maintained constant, owing to a balance between cell exfoliation at the villus tips and proliferation and maturation of the cells in the crypts. The process of maturation, migration, and extrusion requires approximately 3-6 days (Granger et al. 1985).

2.3.4 DEGRADATION OF MUCIN

In the small intestine, it has been shown that neither bile nor luminal acid have an effect on mucus degradation (Allen et al. 1984, Bell et al. 1985). Exhaustive proteolysis has been shown to be associated with the loss of about 20-30% of amino acids except for

threonine, serine and proline, and little (<1%) or no loss of carbohydrate, resulting in the ileal recovery of mucin of more than 95% (Mantle and Allen 1981, Mantle et al. 1981, Scawen and Allen 1977). Hoskins (1981) and Variyam and Hoskins (1983) postulate that at least 50% of the carbohydrates must be removed before the onset of native protein degradation. Oligosaccharide sidechains are believed to protect susceptible bonds of the protein of native mucin, making it resistant to proteolysis until their removal. In addition, mucus gels contain a large amount of contaminant materials including lipids and proteins which have been demonstrated to strengthen the gels, thus making them more resistant to proteolysis (Lee et al. 1987, Sellers et al. 1983). Therefore, it is assumed that mucin subunits are largely undigested prior to the large intestine where they may be degraded (Forstner and Forstner 1986, Hoskins 1984, Variyam and Hoskins 1983).

Clamp and Gough (1991) found that human ileostomy effluent dry matter consisted of about 15% glycoproteins but that feces contained only trace amounts, suggesting that the degradation of mucin occurs mainly in the large intestine. In autopsied humans, analysis of luminal contents revealed regional differences in mucin constituents that were consistent with that seen in microbial degradation (Vercellotti et al. 1977). A variety of glycosidases produced by enteric bacteria are required for the complete degradation of mucin oligosaccharide chains. These enzymes are primarily extracellular exoglycosidases that cleave terminal monosaccharides one at a time from the non-reducing end of the oligosaccharide chains. A high degree of substrate specificity is defined by the monosaccharide to be cleaved, its anomeric configuration and the location of its glycosidic linkage to the next sugar (Hoskins 1984). A combination of bacterial species,

each requiring a separate set of enzymes, may be needed for the complete degradation of mucin glycoproteins. However, only a small portion (about 1%) of the 300-500 taxonomically distinct types of bacteria found in feces are capable of degrading mucin glycoproteins (Bayliss and Houston 1984, Miller and Hoskins 1981, Stanley et al. 1986).

Although mucins are degraded in the large intestine, whether or not the threonine released as a result of mucin degradation is absorbed by the large intestine and then used by the body is not known. If this threonine is absorbed this means that threonine used in mucin synthesis is not lost and that absorption is only delayed relative to intake. Therefore, short term experiments may overestimate the true dietary requirement (Chen 1997, Stoll 1998). However, if threonine is not absorbed by the large intestine, then the threonine used in mucin synthesis would be unavailable to the rest of the body.

Research by Schmitz et al. (1991), based on the injection of homoarginine into the cecum, suggests that the absorption of amino acids in the large intestine may attribute less than 3% of the total amino acid requirement of the pig. Zebrowska et al. (1977) have shown that protein or amino acids infused discontinuously into the cecum have little or no protein value as the nitrogen was almost entirely excreted in the urine. Kidder and Manners (1978) have also observed that the amount of nitrogen absorbed in the hind gut for protein synthesis in the pig to be very low or negligible. The common explanation for this is that protein and amino acids entering the hind gut are converted to ammonia or amines which are absorbed but cannot be used for protein synthesis (Just et al. 1981, Mason et al. 1976, Sauer 1976, Zebrowska 1973).

In the newborn pig, unidirectional fluxes of different amino acids (including threonine)

have been determined across both the small intestine and colon (Smith and James 1980). As well, the systems responsible for amino acid transport are present in the same proportion in both tissues. However, the colonic transport of amino acids occurs for only the first day or two of life (Wooding 1978). This seemingly transient overspill function of the small intestine is thought to be due to the absence of intestinal differentiation at the ileocecal junction immediately after birth. After one day however, the piglet colon show no structures equivalent to the villi of the small intestine. Thus, threonine absorption in the large intestine of pigs greater than two days of age for use by the body probably does not occur. Therefore, the threonine that is used for mucin synthesis is not absorbed and needs to be replaced by the diet. Furthermore, short-term estimates of threonine requirement is accurate.

2.3.5 ACIDIC AND NEUTRAL MUCINS

In the structure of mucin, the protein core is attached to three main types of oliosaccharide side chains. Two of these are acidic (N-acetyl neuraminic/sialic acid, N-acetyl-galactosamine-6-sulphate) and the third is of neutral pH (hexose aldehydes) (Lamont 1992, Spicer and Schulte 1992). The acidic mucins are rich in proline, threonine and glycine while the neutral mucins are rich in serine, aspartate, alanine. (Wesley et al. 1985). The synthesis and sulphation of mucins occurs in the cytoplasm which is compressed towards the basolateral margins of the goblet cell by the central accumulation of large secretion droplets (Lane et al. 1964). Central granules may be synthesized early in the life of a cell and may remain unmoved or unsecreted during most of its lifespan in

colonic crypts (Specian and Neutra 1982). Acidic mucins contain the carbohydrates hyaluronic acid and chondroitin sulphate. Hyaluronic acid is very hydrophilic and absorbs water to form the mucus gel. This gel in turn protects the mucus membrane by binding pathogenic bacteria, parasites (and their toxins) and arresting their movement (Leppi and Stoward 1965, Magnusseon and Stjernstrom 1982). Streptococci and pneumococci contain the enzyme hyaluronidase which determines their pathogenicity. Hyaluronic acid in mucin acts as a substrate and thus may buffer the action of hyaluronidase (Avtsina 1971, Linker et al. 1956). Alterations in the sialylation and sulphation of mucins and in the O-acetylation of mucin sialic acids result in important effects on the resistance of mucus to bacterial enzymatic challenges (Rhodes 1989). O-acetylation normally confers resistance to bacterial sialidases and colitis mucins are therefore more susceptible to degradation by sialidases (Reid et al. 1985).

Terminal sialic acid residues from secreted mucosal goblet cell mucins serve specific protective functions by acting as a substrate for neuraminidase (sialidase). This enzyme has been isolated from bacteria such as *Vibrio* cholerae (Stetskova 1961, Warren and Spicer 1961), *Clostridium perfringens* (Gottshalk 1958), and viruses such as the influenza virus C and paramixyl viruses (Gottshalk 1958, Herrler et al. 1992). In addition, sialic acid residues are also important in the ligand recognition of the gastroenteritis coronavirus which depends upon α2,3-linked sialic acid on the erythrocyte surface. It is believed that interactions between sialic acid and hemagglutinin induced by the virus are believed to be integral in the binding of the virus to the intestinal mucosa (Schultze et al. 1996). Furthermore, the malaria parasite *Plasmodium falciparium* adheres to cells via

sialic acid of erythrocytes. Chinese hamster ovary (CHO) cells treated with neuraminidase showed a 50% reduction in parasite binding (DeLuca et al. 1996).

Bacterial degradation of the mucus gel occurs by way of enzymes such as glycosidases and sulphatases. Bacteria avidly bind to sulphomucins which are then acted upon by sulphatases (Hoskins 1978). The excess erosion of the mucus gels may eventually lead to ulcer formation. For example, *Helicobacter pylori* infections, which are associated with gastritis, gastric ulcers and gastric cancer, are dependent in the oral cavity upon sulphated mucins as receptors (Veerman et al. 1997). Also, gram-negative *Escherichia coli* strains bind preferentially to O-glycans of glycophorin A (i.e. sulphomucins) and are then dispelled by peristalsis (Liukkonen et al. 1992).

Not much is known regarding the specific functions of the neutral mucin sub-type but there is some indirect evidence that its presence protects the mucin protein core and mucosa from proteolysis and at the same time protects the mucosa from bacterial and pancreatic proteases (Lamont 1992). In immunized rats challenged with the nematode *Nippostrongylus brasiliensis*, goblet cell hyperplasia (increased number) of neutral mucins was observed and most of the parasites remained in the lumen entrapped in globules of mucus. Primary worm infection results in a specific *N. brasiliensis*-specific IgE response with goblet cell hyperplasia. Reinfection resulted in the release of mediators such as histamine and serotonin which triggered a massive release of goblet cell mucus (Miller et al. 1981, Wells 1963).

Therefore, alterations in the normal pattern of sulphation and sialylation of intestinal mucins may have dramatic effects on the ability of the mucosal lining of the gut to prevent

the invasion by pathogens.

2.3.6 SPECIES DIFFERENCES IN LOCALIZATION OF MUCINS

Neutral, sialylated, and sulphated mucins have been found to exist in the small intestine and colon of several species, including pigs (Belanger 1963, Rakho and Saloniemi 1972), rabbits (More and Bayle 1972), dogs and cats (Sheahan and Jervis 1976), rats (Katsuyama and Spicer 1978) and humans (Ganter and Marche 1970). However, the distribution of these mucins may vary from one species to another and from one part of the gastrointestinal tract to another. For example, stomach mucosal mucins and mucins in Paneth cell granules are mainly neutral while small intestinal and upper colonic crypt mucins are mainly sialylated. Colonic mucins of sheep contain both sialic acid and sulphate residues (Carson 1990).

In conventionally reared rats, neutral mucins predominate in the small intestine while acidic mucins predominate in the large intestine. Furthermore, goblet cells in the upper part of the large intestinal crypts contain more sulphated mucins than those in the lower crypts (Sharma et al 1995). This duality is also seen in the lung of the rat where new goblet cells developing in the peripheral airways appear to go through a phase of developing sialomucins and later sulphomucins (Lamb and Reid 1968). In addition to these differences in Alcian Blue (AB) staining, changes in PAS (periodic acid/Schiff base) reactive neutral mucins have been observed during the upward migration of goblet cells along the crypts in the intestinal tract of the chick, mouse, and rabbit (Sharma et al. 1997). In contrast to the rat, cecal goblet cells of the chick are devoid of N-

acetylgalactosamine and α-L-fucose residues, indicating a lack of sulphated mucins altogether (Sharma et al. 1997).

The pattern of distribution of neutral and acidic mucins in the pig intestine holds several similarities to that of other species. In the pig intestine, intracellular segregation of apical acid and basal neutral mucins is the same as that reported in rat airway surface epithelium by Jones in 1976. In weaned and unweaned pigs, sulphated mucins predominate at all sites of the intestinal tract, particularly in the large intestine, while O-acetylated sialomucins predominate in larger pigs (60 kg) and humans (Lapertosa et al. 1984, More et al. 1987). Migration of goblet cells up the crypts and villi in the small intestine is associated with an increased production of sulphated mucins. In both the small and large intestine, non-sulphated sialomucins are found mainly at the base of the crypts, which is similar to the distribution in the cecum and ascending colon of humans (Lapertosa et al. 1984).

Brown et al (1988) described the location of mucins in the pig intestine. Sialylated mucins were found at the apex of the cell and sulphated mucins basally, opposite to that found in the rat. Neutral mucins in the pig were found in goblet cells in the crypts and villi, either solely or together with acidic mucins and increased in abundance from the base toward the villus tip. In the small intestine, staining by PAS/AB revealed a majority of goblet cells stained purple. The degree of sialomucins (blue, acidic) decreased towards the tips of the villi and cells stained by PAS alone (red, neutral) occurred less frequently at all levels of the crypt and villus. Cells containing discrete areas of red and blue were found mainly in the region of the base of the villi and the top of the crypts. In the cecum,

sialomucin staining was found to be greatest in the crypts and decreased towards the surface while sulphated mucins increased towards the surface of the villi. In the colon and rectum, the lower crypt goblet cells were mainly blue-staining sialomucins, with only a few red and purple stained cells. Red-staining neutral mucins increased in number progressively toward the upper crypt. In the one-day old piglet, no goblet cells were stained when treated for sialomucin staining (Brown et al. 1988).

In the lower small intestine of weaned pigs, goblet cells were found to be more numerous than in unweaned pigs (Brown et al. 1988). No changes were noted in the different staining characteristics of cells in the sections of upper and lower small intestine stained with PAS/AB 2.5 at any time after weaning on a diet of soya protein. In the large intestine (cecum, colon, rectum) no changes were noted in the number of, or staining characteristics of goblet cells in pigs at any time after weaning. Also, there was a temporary increase in sulphated mucins, particularly in the crypts after weaning. In the unweaned pig, the downward migration of goblet cells from the mid-crypt region was not found to be associated with sulphated mucins (Brown et al. 1988). Perhaps the rise in sulphated mucins are due to increased mucus production by the gastrointestinal tract due to the consumption of solid food, by a hormonal trigger from the absence of sow's milk, or due to threonine deficiency immediately post-weaning.

These findings are in contrast to the mouse, where intestinal epithelial mucus cells first appear in the mid-crypt region, divide and migrate both upwards towards the villus and downwards into the deeper regions of the crypts (Cheng and Bjerknes 1980). Migration of goblet cells up the crypts and villi in the small intestine of unweaned pigs was found to

be associated with an increased production of sulphated mucins, perhaps reflecting the maturation of the goblet cell.

In humans, immature goblet cells deep within the crypts in the small intestine produce neutral mucin with little sialic acid. As the maturing cells migrate, the mucins become more sialylated. The sialic acid residues not only increase the acidity of the molecule but also are sites for further modification by N- and O-acetylation (Filipe 1979). The jejunum and colon of both pigs and humans synthesize O-acetylated sialomucins, with a higher concentration of these mucins in the jejunum (Fischer et al. 1984, Reid et al. 1984). N-acetylated neuraminidase-susceptible sialomucins in the pig were only detected at low levels in the colon because O-acetylation predominates (More et al. 1987), while N-acetylated compounds were found to be only partially labile to neuraminidase (Culling et al. 1974).

In the human colon, goblet cells within the crypt contain mainly sulphated mucins while the mature goblet cells on the colonic surface contain neutral mucins (Lapertosa et al 1984). Lectin cytochemistry has shown that in humans and the CF-1 mouse, mucins at the crypt base of the colon contain more N-acetylglucosamine and less terminal fucose than the more mature cells of the upper crypt. Also, these mucins have non-reducing terminal galactose residues that are absent from mucins produced in mature goblet cells (Boland et al. 1985). Therefore, altered glycosylation patterns accompany the maturation process but it is unknown whether these changes are occurring with the core peptides (Specian and Oliver 1991).

In the human fetal small intestine, mucin droplets around the seventh week have been

found to contain only PAS-positive neutral mucins and that sialidase-labile sialomucins appear later in development (Lev et al. 1972). This would suggest that neutral mucins are produced by immature cells in the crypts, with sialomucins being synthesized as cells mature and migrate towards the villi.

Although the different sub-types of gastrointestinal mucins are all present in the species reviewed, there are some important dissimilarities. Specific localization of acidic and neutral mucins occur within the villus, and the pattern of distribution of these sub-types differ among different species. Therefore, any review and interpretation of experimental findings must take into account the species of animal used and conclusions may not necessarily be applicable across species.

2.3.7 MUCINS AND DISEASE

The mucus layer, covering most of the tissue surface, is an environment richly populated by bacteria and protozoa and can contribute to inhibiting microbial overgrowth (Specian and Oliver 1991). The capacity of mucin-type glycoproteins to form a protective gel and to interact with bacterial species depends largely on their content of oligosaccharides. Therefore, it is believed that changes in the secretory glycoconjugates result in the impairment of these functions and are directly related to some intestinal diseases (Sharma and Schumacher 1995).

Several mechanisms have been proposed to explain the ability of mucus to protect the underylying epithelium (**Table 2.3.7.1**).

Table 2.3.7.1 Mechanism of Protection by Intestinal Mucins

Mechanism	Example	Species	Reference
Mucus expulsion of parasite in immunized animals	*Nippostrongylus* infection	rat, sheep	Miller et al. 1981, Miller and Nawa 1979, Wells 1963
Inhibition of bacterial binding	cholera, pathogenic *E. coli*		Laux et al. 1984, Sajjan and Forstner 1990
Increased mucus release	cholera	rat	Forstner et al. 1981, Mantle et al. 1989
Inhibition of toxin binding	cholera toxin	pig	Stromback and Harrold 1974
Quenching of oxygen radicals	gastric mucin	pig	Grisham et al. 1987

from Lamont (1992)

The simplest mechanism suggests that mucus provides a physical barrier or blanket approximately 400 μm in thickness which physically traps bacteria and viruses and prevents their attachment to the apical surface. Another possibility is that the polysaccharides of mucus-type glycoproteins contain identical carbohydrate sequences found on membrane receptors for bacteria or toxins. These soluble receptors bind to bacteria and viruses and prevent their attachment to the cell surface. A third potential protective mechanism involves the accelerated secretion of mucin which engulfs pathogens and carries them from the epithelial surface (Lamont 1992).

Mucins are believed to bind and trap pathogens by two distinct methods. First, certain carbohydrate moieties on the mucin molecule can immobilize pathogens within the mucus

layer by either mimicking epithelial cell membrane glycoproteins that are recognized and bound by a pathogen's adherence to lectin or by binding to other membrane components, such as type 1 pili expressed by Escherichia coli. Secondly, mucus is a reservoir of IgA, the first line of defence against pathogens such as bacteria (and their toxins) and viruses (Lamont 1992, Magnusseon and Stjernstrom 1982, Oliver et al. 1989). Interactions between secretory immunoglobulin A (IgA) and mucins trap IgA-coated bacteria within the mucus layer, preventing bacterial binding to the brush border membrane. Subsequent degradation and renewal of the mucus layer, coupled with peristalsis, results in the expulsion of trapped pathogens (Krogfelt et al. 1991, Lake et al. 1980, Specian and Oliver 1991, Wang and Andersson 1994). Mucins also function to protect the epithelium from digestion by acting as a substrate for degradative enzymes produced by intestinal flora. Partially purified enzyme fractions of fecal extracts contain α-galactosidase, β-N-galactosaminidase, sialidase, β-glucuronidase, blood-group-degrading enzymes, and proteases (Variyam and Hoskins 1981). These enzymes are present in anaerobic fecal cultures and have been demonstrated in vitro to remove >90% of the total carbohydrate from mucins (Specian and Oliver, 1991).

2.4 DIETARY EFFECTS ON MUCOSAL GROWTH AND MUCINS

As with all other organs, diet can greatly affect the growth of the gastrointestinal mucosa. Since threonine is an important constituent of the mucus gels lining the epithelial surface, any dietary changes in the intake of this essential amino acid would likely affect its incorporation into gut mucins. This would ultimately affect the gut's ability to form

mucus gels, resulting in alterations in the way the body is protected against ingested pathogens.

2.4.1 DIETARY EFFECTS ON MUCOSAL GROWTH

In general, there are three types of stimulation that result in gastrointestinal mucosal growth: a) the numerous processes brought into action by the ingestion and digestion of food; b) gastrointestinal hormones; and c) non-gastrointestinal hormones such as thyroxine and growth hormone. The importance of food was noted by Steiner et al. (1968), who demonstrated in rats starved for 6 days that the small intestine lost 53% of its weight while whole-body weight loss was only 32%. They further found that the total intestinal cell population decreased and the RNA, protein, and water content of the individual cells was diminished compared to that in non-gastrointestinal mucosal tissues.

The effects of food are divided into direct and indirect effects. Direct effects include exfoliation, local nutrition, and direct stimulation of growth by particular dietary constituents that act as growth factors independently of their nutritive value. In the small intestine, the mitotic rate is regulated through a negative feedback system involving chalones that ensure that lost cells are replaced by equal numbers of new cells. Exfoliation removes chalones and their negative influence on dividing cells. Thus, the increase in loss of cells following a meal is one possible explanation for the postprandial increase in mucosal cell renewal. Local nutrition refers to the absorption of digestion products by mucosal cells prior to the nutrients being circulated systemically. Food may also contain compounds that are direct stimulants of mucosal growth or are converted to

trophic factors by the action of digestive enzymes or bacteria within the lumen (Siponen et al. 1976).

Indirect effects of food include those caused by gastrointestinal peptides, increased motor or secretory activity, and nerve stimulation, all of which are interrelated since peptides and nerves regulate secretion and motility, and nerves may release peptides. The gastrointestinal peptides can be separated into their paracrinal, endocrinal, and neurocrinal hormone effects. For example, Holst et al. (1983) demonstrated the inhibition of gastrin release by somatostatin acting as a paracrine hormone. Several gastrointestinal peptides may act as neurotransmitters, including vasoactive intestinal peptide (VIP), bombesin, and enkephalin. VIP is released by vagal stimulation and may be responsible for mediating neurally stimulated relaxation of gut smooth muscle (Furness et al. 1981). Bombesin (gastrin releasing peptide in mammals) appears to be the vagal or neural mediator of gastrin release (Sagor et al. 1985). The enkephalins may be involved in the regulation of gut smooth muscle tone and contraction (Straus et al. 1974).

2.4.2 DIETARY EFFECTS ON MUCINS

The presence of nutrients in the intestinal lumen is a major factor influencing bacterial colonization in vertebrates. Intestinal mucins act as a substrate for the resident flora and by aggregation facilitate the removal of pathogenic bacteria (Sharma et al. 1997). The amount and composition of mucins are in a balanced state between the degradation of luminal mucins by bacteria and their renewal by goblet cell secretions from the intestinal crypts. Bacterial enteropathogens must traverse the mucus layer in order to approach and adhere to epithelial cells. Some bacterial species attach to the gut epithelium by fimbriae

which recognize glycoconjugate receptors in mucus and/or brush border membranes (Sharma et al. 1997). Because dietary factors that either affect the production of mucin or enhance its degradation would make the intestinal mucosa susceptible to pathogenic organisms, it may be possible to modulate the colonization of bacteria via the modification of diet and thus protect against infection. Alternatively, inadequate diets may reduce the mucus barrier and increase the likelihood of infection.

In rats deprived of 50% of their daily intake, mucin concentration/mg protein as well as per mg DNA was found to be significantly decreased. Because sucrase activity per mg protein or DNA did not decrease in malnourished rats it was believed that the reduction in mucin concentration was selective and did not reflect all surface glycoproteins. Goblet cells themselves were variably decreased in malnourished rats, supporting the hypothesis that the maintenance of mucus synthesis has a lower priority during malnutrition than the preservation of the digestive absorptive surface of the microvilli (Sherman et al. 1985). Malnutrition thus leads to an absolute decrease in intestinal mucins and may be a factor in modulating intestinal resistance to enteric infection.

In the rat, intestinal mucosa was found to adapt to different diets and microbial populations by modifying mucin composition, as well as crypt-villus architecture and goblet cell glycoconjugates (Sharma et al. 1995). Rats fed a commercial diet (consisting of barley, maize, wheat, soya bean meal and fish meal) were found to have a greater abundance of N-acetyl-glucosamine and sialic acid-linked D-galactose-specific lectins in small intestinal surface goblet cells than in rats fed a purified diet (consisting mainly of casein, maize starch and potato starch). Lectins are used as histochemical probes to

analyze the changes in the expression of carbohydrate residues in goblet cell mucins in response to diets. Similar increases were seen with a N-acetylgalactosamine-specific lectin in the large intestine of rats fed a commercial diet (Sharma and Schumacher 1995). Because galactose, N-acetyl-glucosamine and N-acetylgalactosamine are abundant in the structure of intestinal mucins, it is apparent that dietary constituents can have dramatic effects on mucin synthesis.

Modifications of diet have been shown to affect the amount of neutral and acidic mucins in the gut. For example, broiler chicks at 5 weeks of age fed a wheat-based diet supplemented with 0.1% xylanase exhibited a lower viscosity of jejunal contents versus the control group (Sharma et al. 1997). Xylanase inclusion in the diet also resulted in an increase in neutral mucins in the small and large intestines and more carboxylated and sulphated mucins in the upper crypt cells of the small intestine and cecum. In chicks fed a maize-based diet, neutral mucins were found to be greater in surface and upper crypt goblet cells of the small and large intestines but decreased in the cecum. While no differences in acidic mucins were found between wheat and maize-based diets, neutral mucins were increased in the surface and upper crypt cells of the small and large intestine in maize-fed chicks (Sharma et al. 1997). This suggests that neutral mucins may be more susceptible to dietary changes.

Non-specific nutrients such as dietary fibre may also have an effect on mucins. Pigs (60 kg) fed a fibre-free milk-based diet exhibited decreased staining intensities for intestinal mucins compared to a standard diet containing 14.7% fibre (cellulose, hemicellulose, lignin). The differences were eliminated when bran was added to the milk

diet, indicating that dietary fibre increased the turnover of jejunal mucins (More et al. 1987). Similar results were observed in rats by Vahouny et al in 1985. Furthermore, jejunal sialic acid staining reactivity was lowered in the jejunum but was enhanced in the colon (More et al. 1987). Glycoproteins in the jejunum also became neuraminidase susceptible while glycoproteins in the colon became characterized by the absence of neutral mucins, indicating that dietary fibre also modifies the nature of mucins secreted (More et al. 1987).

2.5 SUMMARY

The preceeding review of the available literature has demonstrated that mucin is important in the gut for the protection of the gastrointestinal epithelium as well as the underlying tissues, and to prevent invasion from pathogenic organisms. Moreover, mucin is affected by changes in the diet and specifically is likely dependent upon the intake of threonine since this essential amino acid is critical to the structure and function of mucin proteins. The data reviewed has also shown that threonine is taken up by the gut to a much greater extent than any other essential amino acid. This suggests that much of the dietary threonine is used within the gut itself. Impaired gut growth and function in parenterally-fed individuals may help explain the difference in dietary threonine requirement between oral and intravenous feeding. An experiment that would define this difference under identical conditions would strongly support the specific need for threonine by the gut.

If dietary threonine can be shown to be required by the gut selectively, it would be of

interest to determine whether on not dietary threonine actually does affect mucin production. Because different types of mucins (acidic, neutral) seem to have different roles in the gut and seem to be affected by diet differently, any study involving the effect of threonine intake upon the amount of mucin produced by the gut should separate these functionally distinct subtypes.

3. THESIS RATIONALE AND OBJECTIVES

The threonine requirement for piglets of approximately 8 days in age has been determined by Chen (1997, thesis) to be 0.20 g/kg/d when fed intravenously, while the oral requirement has been estimated at 0.61 g/kg/d (NRC 1998), a 3-fold difference. In similar studies involving lysine requirement of piglets receiving TPN, the requirement was observed to be similar (0.79 g/kg/d, House et al. 1998) to published estimates (0.86 g/kg/d, NRC 1998). Thus, my first objective is to quantify the threonine requirements of neonatal piglets (fed a diet identical to Chen's TPN diet) intragastrically (into the stomach), in order to determine if a difference in requirement exists.

Experiment 1 Objectives: Given identical diets and methods differing only in route of feeding, my aim is to:

1. Empirically determine the THR requirement for neonatal pigs, since published estimates are extrapolated from data coming from older pigs.
2. Compare the THR requirement determined experimentally to published estimates of THR requirement

Experiment 1 Null Hypotheses:

1. The THR requirement of piglets fed intravenously and piglets fed intragastrically is not different.
2. The THR requirement of neonatal piglets fed orally is not different from published estimates.

Experiment 2 Objectives:

This experiment will focus upon the effect of THR on gut mucins, due to the importance of THR in the structure and function of mucin and the importance of mucins on gut health, growth and digestion. Specifically, my aim is:

1. To determine if THR intake has an effect on gut mucins.

Experiment 2 Null Hypotheses:

1. The quantity gut mucins in piglets receiving a THR-deficient diet vs. a THR-adequate diet is not different.
2. The quantity of gut mucins in piglets receiving THR orally vs. intravenously is not different.

4. EXPERIMENT 1: THREONINE REQUIREMENT OF NEONATAL PIGLETS FED INTRAGASTRICALLY

4.1 INTRODUCTION

The goal in feeding human neonates both parenterally and enterally is to provide amino acids at sufficient levels for protein synthesis. At the same time, excess intake is avoided as surplus pools of amino acids and/or their degradative products may stress immature enymatic removal processes, leading to toxicity. High plasma threonine concentrations may be problematic in infants due to its possible neurotoxic effects, as threonine levels in cerebrospinal fluid increase with increases in plasma threonine concentration. This may impair the proper development of the central nervous system in the neonate (Anderson and Raiten 1992).

In both preterm (Schanler and Garza 1987) and term infants (Janas et al. 1985), threonine intake was found to correlate with plasma concentrations, suggesting the oxidation of excess threonine was insufficient. Also, the highest threonine concentrations were seen in infants of the lowest gestational age (Rigo and Senterre 1980). In growing rats, hepatic threonine dehydratase activity was found to be low during the postnatal period in contrast to its activity in adult rats (Grogan et al. 1988).

The threonine requirement of piglets determined from the intravenous infusion of a purely synthetic diet was found by Chen. (1997) to be 0.2 g/kg/d. In contrast, published estimates of threonine requirement is 0.61 g/kg/d (NRC 1998). This large discrepancy may be due to a combination of differences in route of feeding, composition of diet, method of estimation, and the age of pigs observed. Consequently, this experiment

determined an oral threonine requirement that would be more comparable to the intravenous data. Using the same methods as Chen and modifying only the route of feeding to intragastric infusion would eliminate the systematic discrepancies between Chen's results and that of the NRC. A true comparison of intravenous and oral threonine requirement in the neonatal piglet model for premature human infants would then be possible. The objectives of this experiment are to empirically deteremine the intragastric threonine requirement for neonatal piglets and to compare this requirement with published estimates based upon extrapolations from older pigs. The null hypothesis is that the threonine requirement of piglets fed intravenously and intragastrically is not different, nor is this requirement different from published values.

4.2 MATERIALS AND METHODS

4.2.1 ANIMALS AND SURGERY

Twenty-six male Yorkshire piglets (approximately 2-3 days of age) were assigned to one of 7 threonine treatments (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 g/kg/d) in a completely randomized design. On day 0 of the study, the piglets were transferred from a specific pathogen-free herd (Arkell Swine Research Station, University of Guelph) to the animal holding facilities in the Department of Animal Science, University of Guelph. All piglets had remained with the sow prior to the study. The piglets were initially anesthetized for surgery by injection of acepromazine (0.5 mg/kg Atravet™, Ayerst Laboratories, Montreal, PQ) and ketamine hydrochloride (22 mg/kg Rogarsetic™, Rogar STB Inc., Montreal, PQ). Subsequent anesthesia was maintained by the administration of a mixture

of oxygen and halothane (Fluothane™, Ayerst Laboratories, Montreal, PQ). Under sterile conditions, a 2 cm (3 mm deep) incision was made in a transverse direction on the left side of the chest. A silastic catheter (1.0 mm i.d. x 2.2 mm o.d.) was tunneled subcutaneously from a point along the initial incision towards the neck region and externalized. A 2 cm sagittal incision was made lateral to the trachea and the left jugular vein was isolated. The silastic catheter was tunneled towards the jugular vein and then inserted and extended to the superior vena cava.

A 2 cm incision was made in the left thigh at the femoral vein. The femoral vein was isolated and a silastic catheter was tunneled subcutaneously from a point along the original incision towards the left femoral vein. The catheter was inserted in the femoral vein and extended to a point proximal to the hepatic artery.

A 3 cm midsagittal incision was then made in the abdomen, extending from the umbilicus. The stomach was externalized and a catheter was inserted and anchored. The free end of the catheter was then tunneled towards a point along the original incision and externalized. All catheters were anchored to surrounding muscle tissue by silastic grommets. All incisions were closed and covered with a topical antibiotic cream (Hibitane Veterinary Ointment™, Ayerst Laboratories, Montreal, PQ). Catheter positions were verified at necropsy and catheter patency was maintained by flushing with saline daily. Post-operative intramuscular injections of 0.4 cc sterile antibacterial sulphadoxine (Borgal™, Hoechst Roussel Vet Canada Inc, Regina, Sask.), and 0.7 cc gentamicin sulphate (Garasol™, Scherring Canada Inc., Pointe-Claire, Que.) were then administered. The piglets were then placed in individual cages in banks of four and fitted

with a vest attached to a tether and swivel (Alice King Chatham Medical Arts, Los Angeles, CA) located at the top of the cage. This allowed for visual and aural contact with litter-mates as well as complete freedom of movement of the piglet and ease of feeding. The cages were maintained in an isolated room at 25°C with supplemental heat provided by heat lamps. The room was kept on 12 h light, 12 h dark cycles and toys placed in the cages were used to enrich the environment.

4.2.2 NUTRIENT SOLUTIONS

Following surgery (Day 0), the piglets were fed intravenously via the jugular catheter for about 24 hours at a rate of 6.75 mL/kg/h (half normal rate). On Day 1, adaptation to oral feeding commenced and feeding was administered via the gastric catheter at a rate of 20.25 mL/kg/h at a dilution ratio of 1:2 (nutrient solution : H_20) for 12 hours. The dilution ratio was then changed to 1:1 (nutrient solution : H_20). On day 2, the dilution was changed to 2:1 (nutrient solution : H_20) and maintained for the duration of the study.

The piglets received the base nutrient solution **(Table 4.2.2.1)** for a period of 5.5 days containing a threonine level of 0.8 g/kg/d (1.64 g/L diet), followed by the introduction of the test nutrient solution containing varying levels of threonine and alanine. Alanine levels in the nutrient solution were altered to maintain an isonitrogenous diet. On day 6, an indicator amino acid oxidation study was performed, with the intragastric infusion of the test nutrient solution being maintained for the duration of the oxidation study.

Table 4.2.2.1 Nutrient Profile of diet used during the initial 5.5 days of the study

Nutrient	Concentration as fed (/L)
L-Alanine	3.29 g
L-Arginine	1.88 g
L-Aspartate	1.88 g
L-Cysteine	0.45 g
L-Glutamate	3.25 g
Glycine	0.76 g
Glycyl-L-Tyrosine	0.72 g
L-Histidine	0.96 g
L-Isoleucine	1.43 g
L-Lysine•HCl	2.19 g
L-Methionine	0.60 g
L-Phenylalanine	0.98 g
L-Proline	2.56 g
L-Serine	1.73 g
L-Taurine	0.14 g
L-Threonine	1.64 g
L-Tryptophan	0.66 g
L-Tyrosine	0.24 g
L-Valine	1.64 g
Dextrose	50.55 g
K_2HPO_4	0.88 g
KH_2PO_4	0.61 g
Potassium acetate	0.82 g
NaCl	1.21 g
$MgSO_4•(7H_2O)$	0.44 g
$ZnSO_4•(7H_2O)$	0.05 g
$MnSO_4$ (1.65 % w/v)	73.5 µL
Calcium gluconate	3.58 g
Micro +6® trace element mix[a]	9.2 mL
MVI® vitamin mix[b]	9.2 mL
Fe dextran (8 % v/v)	3.1 mL
Intralipid 20%®[c]	97.5 mL
Water (ddH_2O)	complete to 1 L

[a] Contents of Micro +6® trace element mix

Trace Element	Concentration (μmol/mL)
Chrome	0.076
Copper	6.3
Iodine	0.47
Manganese	1.8
Selenium	0.25
Zinc	44.9

[b] Contents of MVI® vitamin mix

Vitamin	Concentration
Vitamin A	2300 U
Thiamine (as hydrochloride)	1.2 mg
Riboflavin (as phosphate)	1.4 mg
Niacinamide	17.0 mg
d-pantothenic acid	5.0 mg
Pyridoxine hydrochloride	1.0 mg
Biotin	20 μg
Folic acid	0.14 mg
Vitamin B_{12}	1 μg
Ascorbic acid	80 mg
Vitamin D	400 U
Vitamin E	7.0 U
Vitamin K_1	0.2 mg

[c] Contents of Intralipid 20%® intravenous fat emulsion

Constituent		Concentration
Soybean oil		20%
Linoleic acid	52%	
Linolenic	8%	
Oleic acid	22%	
Palmitic acid	13%	
Stearic acid	4%	
Egg phospholipids		1.2%
Glycerin (anhydrous)		2.2%

4.2.3 INDICATOR AMINO ACID OXIDATION

The piglets were placed in individual an airflow-controlled (20 L/min) Plexiglas respiration chambers (60 x 40 x 40 cm) and acclimated for 30 min. A tracer solution containing 2.5 μCi/mL L-[1-^{14}C]-phenylalanine (American Radiolabeled Chemical, Inc, St. Louis, MO) was infused at a priming dose of 5 μCi/kg for 10 min., followed by a constant infusion of 3.5 μCi/kg/h for the duration of the 6-hour oxidation study. The tracer solution was infused into the jugular catheter through pressure-sensitive microbore tubing (IVAC, Eli Lilly Inc., San Diego, CA), and the rate of infusion was controlled by a calibrated syringe pump (IVAC Pump Series 700, Eli Lilly Inc., San Diego, CA).

Phenylalanine oxidation was measured by the complete collection of $^{14}CO_2$ eliminated by the piglet (Ball & Bayley, 1984). The air that was pumped from the chamber was bubbled through three gas washing bottles connected in series. The bottles contained 150, 150, and 100 mL, respectively, of a mixture of ethylene glycol monomethyl ether and ethanolamine absorber solution (2:1 vol/vol; Fisher Scientific, Nepean, ON) that was used to trap all of the $^{14}CO_2$ expired by the piglets. The first breath collection period ended 45 min after the initiation of tracer infusion. The gas washing bottles were replaced with clean bottles containing fresh absorber solution. The volume of absorber solution in each bottle was measured at the end of each collection period. A 1 mL aliquot was taken from each bottle for scintillation counting (5 mL AtomLight™ liquid scintillant, Du Pont Canada, Mississauga, ON). Subsequent breath collection occurred at 30 min intervals, with the last collection at 5h, 45 min.

Blood samples (1.5 mL) were taken via the femoral catheter just prior to the

commencement of tracer infusion and continued at 30 min intervals, with the last sample taken at 6h. The blood samples were drawn into heparinized syringes, transferred to 1.5 mL microcentrifuge tubes (Bio-Rad Laboratories, Mississauga, ON), and centrifuged for 3 min at 5000g (Biofuge, Heraeus Instruments, Canlab, Mississauga, ON). The plasma layer was removed and stored at -80°C for later analysis of plasma specific radioactivites of phenylalanine and tyrosine.

At the end of the oxidation study, the piglets were killed by injection of sodium pentobarbital (0.3 mL/kg). Final body weight was measured and the liver was extracted, weighed, frozen in liquid nitrogen, and stored at -80°C.

4.2.4 $^{14}CO_2$ ANALYSIS

The aliquots taken from each absorber bottle were counted for 10 min on a liquid scintillation counter (LS6000SC, Beckman Instruments Canada Ltd., Mississauga, ON), with corrections for background counts as well as counting efficiency (Auto DPM mode).

4.2.5 PLASMA PHENYLALANINE AND TYROSINE CONCENTRATION AND SPECIFIC RADIOACTIVITY

Plasma phenylalanine and tyrosine concentrations were determined by reverse phase high performance liquid chromatography (HPLC), following the method of Bidlingmeyer et al. (1984) for the preparation of phenylisothiocyanate (PITC, Pierce, Rockford, IL) derivatives for HPLC amino acid analysis. 400 μL plasma were mixed with 40 μL internal standard (2.5 μm/mL norleucine, Sigma Chemical Co., St. Louis, MO) and 1 mL

protein-precipitating agent (0.5% trifluoroacetic acid in methanol), and then centrifuged for 5 min at 5000g. The supernatant was collected and freeze-dried. 100 µL of a solution containing triethylamine, methanol, and distilled de-ionized water (1:1:3) was then added and the mixture was vortexed and freeze-dried. 50 µL of a derivitizing solution containing distilled de-ionized water, triethylamine, methanol, and PITC (1:1:7:1) was added and the mixture was vortexed, allowed to stand for 35 min, and then freeze-dried. The derivatives were resuspended in 200 µL sample diluent (5% phosphate buffer in acetonitrile) and 50 µL was injected onto a 30-cm C_{18} reverse-phase HPLC column (Novapak, Waters, Milford, MA) maintained at 46°C, with amino acid elution established by a salt-organic gradient solvent system. An in-line fraction collector (Bio-Rad Laboratories, Mississauga, ON) allowed for the collection of phenylalanine and tyrosine fractions as they were eluted from the column. The fractions (1 mL) were collected into 7 mL plastic scintillation vials (Rose Scientific, Mississauga, ON), biodegradable liquid scintillation cocktail (5 mL, BCS, Amersham Canada, Oakville, ON) was added, and the vials were then counted on a liquid scintillation counter (Canberra-Packard model 1600, Canberra-Packard, Mississauga, ON). The phenylalanine and tyrosine fractions were counted for 40 and 15 min, respectively, with corrections made for background counts and counting efficiency.

4.2.6 CALCULATIONS

Phenylalanine and tyrosine concentrations were calculated as:

[amino acid] ($\mu mol \cdot mL^{-1}$) = (amino acid peak area / Norleucine peak area) · CF · RF,

where CF is the concentration factor (0.25 μmol Norleucine · mL^{-1} plasma) and RF is the response factor for the individual amino acid. Response factors were calculated as:

RF = Norleucine peak area / amino acid peak area

for equimolar standards.

The plasma specific radioactivities (SRA) of phenylalanine and tyrosine were calculated as: **SRA ($dpm \cdot \mu mol^{-1}$) = amino acid radioactivity ($dpm \cdot mL^{-1}$) / [amino acid] ($\mu mol \cdot mL^{-1}$)**

Plasma phenylalanine SRA data was plotted against time and a mean plateau value was calculated from the plasma SRA values during tracer infusion. Plateau values were verified by confirming a zero slope by regression analysis.

Phenylalanine flux of the free amino acid pool was determined by use of a stochastic model of amino acid metabolism described by Waterlow et al. (1978) under steady state conditions:

Flux (Q) = S + E = B + I,

where S = non-oxidative losses, a reflection of protein synthesis

E = oxidative losses

B = protein breakdown

I = intake of phenylalanine

Phenylalanine flux was calculated as:

Q ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) = Dose ($dpm \cdot kg^{-1} \cdot h^{-1}$) / plateau ($dpm \cdot \mu mol^{-1}$),

where Dose = radioactivity of infused tracer.

The rate of $^{14}CO_2$ expiry ($dpm \cdot kg^{-1} \cdot h^{-1}$) from oxidation of the [^{14}C]-Phenylalanine tracer was plotted for each collection period and isotopic steady state was determined and corrected for retention of the label in the bicarbonate pool (bicarbonate retention factor, BRF) as:

Corrected $V^{14}CO_2 \cdot kg^{-1} \cdot h^{-1}$ ($dpm \cdot kg^{-1} \cdot h^{-1}$) = $V^{14}CO_2$ ($dpm \cdot kg^{-1} \cdot h^{-1}$) / BRF,

where $V^{14}CO_2$ is $^{14}CO_2$ production, and BRF = 0.93 (Wykes et al., 1994).

The rate of oxidation relative to the rate of isotope infusion was represented as % dose oxidized and calculated as:

% Dose oxidized = Corrected $V^{14}CO_2 \cdot kg^{-1} \cdot h^{-1}$ ($dpm \cdot kg^{-1} \cdot h^{-1}$) /

Dose ($dpm \cdot kg^{-1} \cdot h^{-1}$) · 100%

The oxidation rates of phenylalanine were calculated as:

Phenylalanine oxidation (E) ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) =

Corrected $V^{14}CO_2 \cdot kg^{-1} \cdot h^{-1}$ ($dpm \cdot kg^{-1} \cdot h^{-1}$) / phenylalanine SRA ($dpm \cdot \mu mol^{-1}$).

The remainder of the flux components were calculated as follows:

Non-oxidative losses (S) ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) = Q - E

Intake (I) ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) = [phenylalanine in diet] ($\mu mol \cdot mL^{-1}$) ·

infusion rate of diet (day 6) ($mL \cdot kg^{-1} \cdot h^{-1}$).

Contribution from protein breakdown (B) ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) = Q - I

Net phenylalanine loss/gain was represented as the apparent phenylalanine balance and calculated as:

Phenylalanine balance ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) = I - E

Conversion of phenylalanine to tyrosine was calculated as:

Tyrosine SRA : Phenylalanine SRA (%) = [^{14}C]-Tyrosine plateau / [^{14}C]-Phenylalanine plateau · 100%

4.2.7 STATISTICS

The THR requirement was determined using breakpoint analysis by two-phase linear regression (SAS v.6.07, Cary, NC). Statistical analyses of THR kinetics were performed by using Tukey's multiple comparison test (SAS v.6.07, Cary, NC) to compare between diet treatment groups.

4.3 RESULTS

4.3.1 WEIGHT CHANGES

During the course of the study, the piglets were healthy and active. The average initial weight of the piglets was 1.75 kg (SD = 0.15), the average final weight was 2.57 kg (SD = 0.22), and the average daily gain in weight was 0.15 kg (SD = 0.03) (**Table 4.3.1.1**). Weights and weight changes were not significantly different among the different diet levels.

Table 4.3.1.1 Weight Changes During Indicator Amino Oxidation Study

	Threonine Intake (g/kg/d)								
Weight (kg)	0.1	0.2	0.4	0.6	0.8	1.0	1.2	Mean	SD_{pooled}
Initial	1.69	1.65	1.88	1.80	1.79	1.77	1.63	1.75	0.15
Final	2.53	2.59	2.82	2.53	2.63	2.59	2.32	2.57	0.19
Wt gain/d	0.15	0.17	0.16	0.13	0.14	0.15	0.13	0.15	0.03
n	3	3	4	4	4	3	4		

4.3.2 PLASMA AMINO ACIDS

As threonine intake increased from 0.1 g/kg/d to 0.2 g/kg/d, plasma phenylalanine concentration declined from 131 μmol/L to 82 μmol/L ($P<0.05$) (**Table 4.3.2.1**). From threonine intakes of 0.2 g/kg/d to 1.2 g/kg/d, plasma phenylalanine concentration was not significantly different. Plasma tyrosine levels declined from 114 μmol/L (at 0.1 g/kg/d of threonine) to 29 μmol/L (at 0.4 g/kg/d of threonine) ($P<0.05$). With increasing threonine intakes, the plasma threonine concentration rose from 34 μmol/L (at 0.1 g/kg/d of threonine) to 1734 μmol/L (at 1.2 g/kg/d of threonine) ($P<0.001$).

Table 4.3.2.1 Amino Acid Concentration in Plasma

	Plasma Concentration (μmol/L) at different Threonine Intakes (g/kg/d)								
Amino Acid	0.1	0.2	0.4	0.6	0.8	1.0	1.2	Mean	SD_{pooled}
Tyrosine	114	76.0	29.4	36.7	33.1	29.9	29.8	41	30
Phenylalanine	131	81.7	71.7	74.3	78.7	66.8	63.5	82	30
Threonine	34.0	36.6	159	652	835	581	1734	612	43

4.3.3 PHENYLALANINE KINETICS

During the oxidation study, plateau levels in both plasma phenylalanine and tyrosine SRA and breath $^{14}CO_2$ were reached 2 h after the initiation of the isotope infusion in all pigs. The ratio of plasma tyrosine SRA:plasma phenylalanine SRA was not found to be significantly between different diet treatments (**Table 4.3.3.1**). Phenylalanine intake for each pig was estimated to be 120 μmol/kg/h. Phenylalanine flux ranged from 416 μmol/kg/h to 515 μmol/kg/h between the different diet treatments. Non-oxidative disposal of phenylalanine among different diet treatments ranged from 358 μmol/kg/h to 494 μmol/kg/h and phenylalanine release from protein ranged from 296 μmol/kg/h to 395 μmol/kg/h. Differences in flux, non-oxidative disposal and release from protein were not found to be statistically significant among diet treatments.

Table 4.3.3.1 Phenylalanine Kinetics

Parameter	Threonine Intake (g/kg/d) 0.1	0.2	0.4	0.6	0.8	1.0	1.2	SD_{pooled}
Corrected $V^{14}CO_2$ ($x10^3$ DPM/kg/h)	1174^{a}	727^{ab}	405^{bc}	272^{bc}	260^{bc}	316^{bc}	244^{c}	191
Plasma PHE SRA ($x10^3$ DPM/umol)	20.4	22.4	19.6	20.9	20.0	19.0	21.0	4.4
Plasma TYR SRA ($x10^3$ DPM/umol)	3.1	2.9	2.8	1.8	1.9	1.7	2.1	1.3
TYR:PHE (%)	5.2	12.8	14.4	8.7	9.4	8.9	10.1	5.8
PHE Flux (Q) (umol/kg/h)	416	439	515	431	482	479	437	107
PHE Oxidation (E) (umol/kg/h)	57.6^{a}	32.5^{ab}	20.7^{b}	13.0^{b}	13.0^{b}	16.6^{b}	11.6^{b}	11.8
Intake (I) (umol/kg/h)	120.0	120.2	119.4	119.4	117.9	118.6	121.3	1.6
Non-ox. losses (S=Q-E) (umol/kg/h)	358	407	494	418	469	462	426	99
Breakdown (B=Q-I) (umol/kg/h)	296	319	395	310	362	359	317	107
PHE Balance (I-E) (umol/kg/h)	62.4^{b}	87.7^{ab}	98.8^{a}	106.5^{a}	104.9^{a}	101.9^{a}	109.6^{a}	11.8
% Dose Oxidized	14.4^{a}	8.1^{b}	4.2^{bc}	3.1^{bc}	2.8^{bc}	$3.5b^{c}$	2.7^{c}	2.1

^{a,b} denote significance by Tukey's multiple comparison test ($p<0.05$)

Phenylalanine oxidation was found to be significantly different across different levels of threonine intake, expressed as either collected $^{14}CO_2$ in breath (**Table 4.3.3.1, Fig. 4.3.3.1a,b**), as calculated with the inclusion of plasma phenylalanine SRA data, or as a

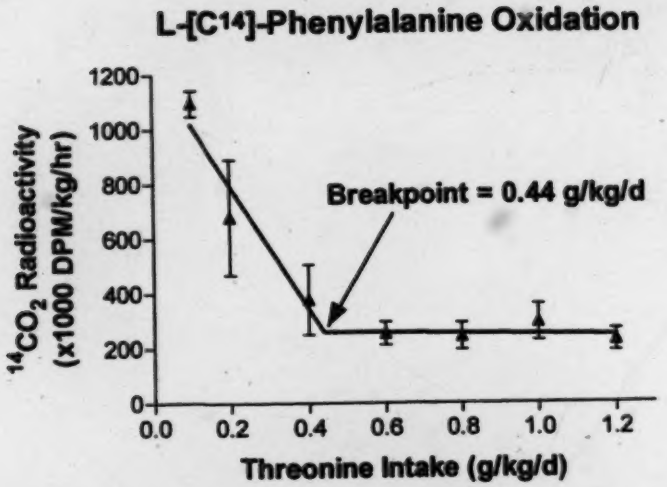


Figure 4.3.3.1a Threonine Requirement by 2-Phase Linear Regression. Mean $^{14}CO_2$ radioactivity in piglets receiving different levels of threonine. Values are means ± SEM.

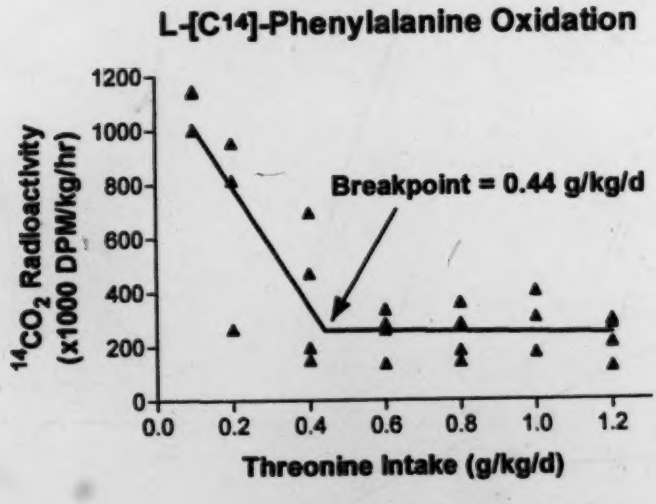


Figure 4.3.3.1b Threonine Requirement by 2-Phase Linear Regression. $^{14}CO_2$ radioactivity in collected breath of individual piglets receiving different levels of threonine.

percentage of the dose oxidized (**Fig. 4.3.3.2a,b**). As threonine intake increased from 0.1 to 0.4 g/kg/d, phenylalanine oxidation declined significantly ($P<0.05$). With subsequent increases in threonine intake from 0.4 to 1.2 g/kg/d, there was no change in phenylalanine oxidation ($P>0.05$). Similarly, apparent phenylalanine balance increased with increasing threonine intakes from 0.1 to 0.4 g/kg/d, to a plateau at intakes >0.4 g/kg/d (**Fig. 4.3.3.3a,b**).

In determining the breakpoint in 2-phase linear regression analysis, the data partitioning that produced the highest regression coefficient was used . The breakpoint, estimated by phenylalanine oxidation rate (**Fig. 4.3.3.1b**) and phenylalanine balance (**Fig. 4.3.3.3b**) occurred at a threonine intake level of 0.46 g/kg/d (95% CI: 0.32-0.61, r^2 = 0.663). The breakpoint estimated by phenylalanine oxidation as a percentage of dose (**Fig. 4.3.3.2b**) was found at a threonine intake level of 0.42 g/kg/d (95% CI: 0.33-0.51, $r^2 = 0.777$). These results are summarized in Table 4.3.3.2.

Table 4.3.3.2 Threonine Requirement by Breakpoint Analysis

Parameter	Breakpoint	Lower 95%	Upper 95%	SE	r^2
$^{14}CO_2$	0.44	0.33	0.55	0.06	0.752
PHE Oxidation	0.46	0.32	0.61	0.09	0.663
PHE Balance	0.46	0.32	0.61	0.09	0.663
% Dose	0.42	0.31	0.51	0.05	0.777

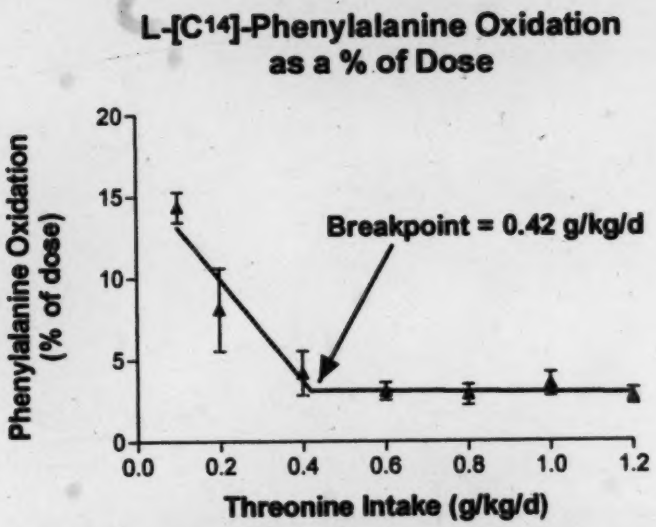


Figure 4.3.3.2a Threonine Requirement by 2-Phase Linear Regression. Mean phenylalanine Oxidation as a % of dose in piglets receiving different levels of threonine. Values are means ± SEM.

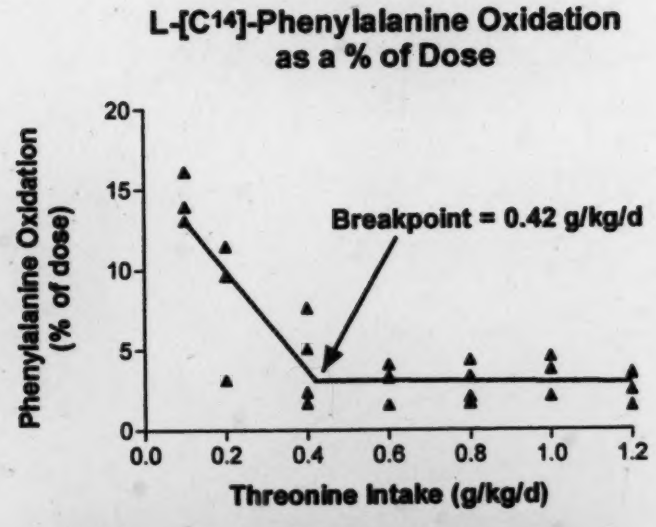


Figure 4.3.3.2b Threonine Requirement by 2-Phase Linear Regression. Phenylalanine Oxidation as a % of dose in individual piglets receiving different levels of threonine.

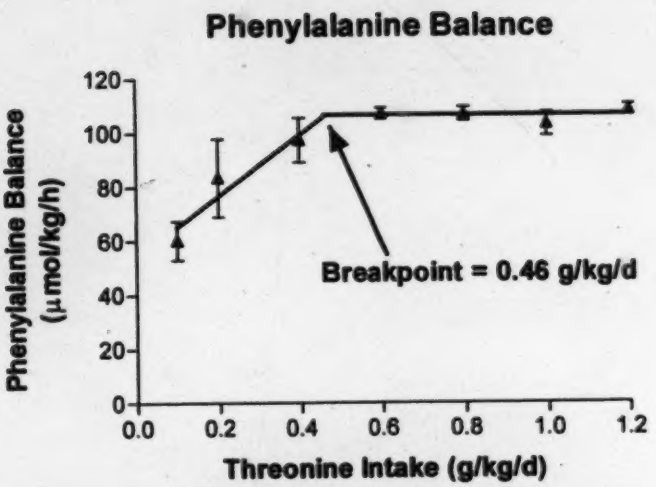


Figure 4.3.3.3a Mean apparent phenylalanine balance in piglets receiving total parenteral nutrition intragastrically with graded intakes of threonine. Values are means ± SEM.

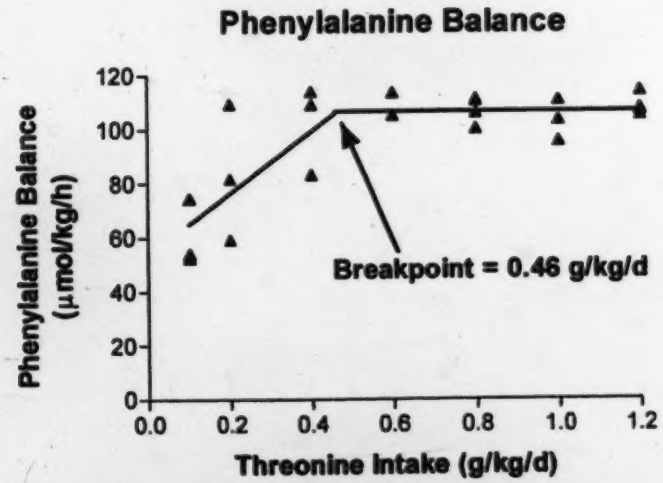


Figure 4.3.3.3b Apparent phenylalanine balance in individual piglets receiving total parenteral nutrition intragastrically with graded intakes of threonine..

4.4 DISCUSSION

The use of the indicator amino acid oxidation technique has been applied successfully in determining amino acid requirements in piglets and humans (Zello et al. 1995). In the case of threonine, this method is especially advantageous due to the complicated nature of its metabolic pathway. As such, direct oxidation techniques are inappropriate or very difficult. During direct oxidation, labeled $^{14}CO_2$ from ^{14}C-threonine would be underestimated due to the sequestration of 1° and 2° carbon atoms into glycine (Ballevre et al. 1990, Le 'Floch 1995). Threonine requirements are thus more suitably estimated by the indicator amino acid oxidation technique.

Several parameters measured in this experiment correlate with data obtained by Chen (1997). The initial and final weights of the pigs, time for the radioactive label to reach plateau in plasma, plasma tyrosine:phenylalanine ratios and plasma phenylalanine changes were similar in both studies. Also, phenylalanine intake, flux, non-oxidative disposal and release from protein were not different among treatments in the present experiment, as was the case in the parenteral study (Chen 1997). In intravenously fed piglets receiving the identical diet (Chen 1997), plasma threonine levels ranged from 88 umol/L at an intake level of 0.05 g/kg/d of threonine, to 647 umol/L at an intake level of 0.6 g/kg/d of threonine. In the current experiment, plasma levels in the intragastrically fed piglets ranged from 34 umol/L to 1734 umol/L at 0.1 and 1.2 g/kg/d of threonine, respectively. At the intravenous requirement of about 0.2 g/kg/d of threonine, the plasma threonine level was about 186 umol/L (Chen 1997). In comparison, at the intragastric requirement of about 0.4 g/kg/d, the plasma threonine level was similar at 159 umol/L. This similarity

in results between Chen's and the current study suggest that comparisons in threonine requirement obtained in each study are comparable, differing only in the route of administration.

The safe level of intake for amino acids would meet the needs of 95% of a normally-distributed population (i.e. the upper 95% confidence interval). The safe level of the requirement of threonine determined by Chen (1997) was 0.21 g/kg/d, while the safe level of threonine determined in the current study was found to be 0.51 g/kg/d. In both cases, phenylalanine oxidation as a percentage of dose was used as the data set was least variable. Using expired CO_2, the requirement level in the current experiment was found to be 0.44 g/kg/d, with a safe level of threonine intake of 0.55 g/kg/d. The similarity in estimates obtained from using different measured parameters (expired CO_2, phenylalanine oxidation, phenylalanine oxidation as a percentage of dose, phenylalanine balance) further exemplifies the efficiency of the indicator amino acid oxidation technique in determining amino acid requirement levels without the need of blood sampling and further isolation of the radioactive label. Similarly, the threonine requirement and safe levels of intake as determined by phenylalanine oxidation and phenylalanine balance were both found to be 0.46 g/kg/d and 0.61 g/kg/d, respectively.

The threonine content of the total parenteral nutrition solution used in the present study corresponds to a threonine intake level of 0.8 g/kg/d. Plasma threonine levels in intravenously fed piglets receiving 0.6 g/kg/d of threonine was 647 umol/L (Chen 1997) while piglets receiving 0.8 g/kg/d intragastrically in the current study had plasma threonine levels of 835 umol/L. Compared to plasma threonine levels at requirement, this

suggests that the threonine content in commercial TPN solutions are much higher than needed and may be nearing a state of toxicity.

Changes in phenylalanine balance represent changes in whole-body protein synthesis. Because balance is defined as Intake - Excretion (by oxidation), an increase in phenylalanine balance would suggest that more phenylalanine is required by the body, presumably for protein synthesis. As the intake of the deficient amino acid (threonine) increases, so too does the body's capacity for protein synthesis, as exhibited by a rise in phenylalanine balance. Above the requirement of the limiting amino acid threonine, protein synthesis is maximal and the difference between the amount of phenylalanine needed for protein synthesis and the amount oxidized as excess amino acid becomes constant.

There is evidence that parenteral amino acid requirements are lower than enteral requirements. In piglets fed parenterally, the lysine requirement was found to be about 72% of the oral requirement for piglets according to NRC estimates (House et al. 1998, NRC 1998). With respect to threonine, the parenteral requirement was found to be only 45% of the oral requirement in piglets fed identical diets under similar environments. Inferences based upon the direct comparison of parenteral data with NRC estimates are unreliable since those estimates are extrapolated from data that is expressed as a percentage of a corn-soybean meal diet (Kim et al. 1983). Also, protein intakes and digestibilities varied widely in those experiments. Thus, the oral threonine requirement determined in this study is more suitable for comparison to data obtained from parenterally-fed experiments.

Lower estimates of amino acid requirements in parenterally-fed piglets might be expected due to the exclusion of first-pass metabolism by the splanchnic organs. The gut is atrophied during parenteral feeding and this fact is of particular significance in the case of threonine as it is highly expressed in the mucus layer that lines and protects the entire gastrointestinal tract. The mucosa of the gastrointestinal tract has one of the most rapid turnover rates of any tissue in the body (Johnson and McCormack 1994), further exhibiting a possible reason for the large disparity in threonine requirement between oral and intravenous feeding. Stoll et al. (1998) found that the gastrointestinal tract metabolizes about 35% of dietary leucine, lysine and phenylalanine on first pass, while 61% of dietary threonine was metabolized. Of this metabolized threonine, 90% of it was either secreted as mucosal proteins or catabolized. In humans, indispensible amino acid losses in the gut has been shown to account for 14-33% of daily maintenance requirement. In the case of threonine this loss accounted for 61% (Fuller et al. 1994). This suggests that threonine may be in high demand by the gut and that a large portion of the body's requirement for threonine is for use by the gut. During parenteral feeding, gut atrophy (i.e. reduced gut function) may help explain the lower threonine requirement versus enteral feeding. Because threonine is highly incorporated in the structure and function of gastrointestinal mucus, specifically mucin proteins (chapter 2), the difference in requirement levels between parenteral and enteral feeding may be explained by its specific use in the gut. During times of gut atrophy, there is a concurrent decrease in the amount of mucus lining the gastrointestinal tract, which may result in a decrease in the amount of threonine required by the gut, and consequently, decrease the body's overall

threonine requirement.

4.5 CONCLUSIONS

The oral threonine requirement in neonatal piglets fed an elemental diet was found using the indicator amino acid oxidation technique to be 0.42 g/kg/d, with a safe level of intake (upper 95% confidence limit) of 0.51 g/kg/d. This corresponds closely to the published mean estimate of 0.6 g/kg/d according to NRC requirements for a corn and soybean meal diet. Although similar quantitatively, an empirical determination is required in order for useful comparisons to our parenteral feeding model to be made. The parenteral threonine requirement, previously determined to be 0.2 g/kg/d, is 45% of the present estimate of the oral requirement. Therefore, while the null hypothesis of no difference in threonine requirements between empirical determination and published estimates is accepted, the null hypothesis that parenteral and enteral threonine requirements are not different is rejected.

5 EXPERIMENT 2: THREONINE AND MUCIN SYNTHESIS IN THE GUT

5.1 INTRODUCTION

The threonine requirement for piglets of approximately 8 days in age was determined by Chen (1997) to be 0.20 g/kg/d when fed intravenously. When piglets were fed an identical diet intragastrically, the requirement level was estimated to be 0.55 g/kg/d (chapter 4), which is similar to NRC guidelines for oral threonine requirement (NRC, 1998). These results suggest that an appreciable portion of the oral threonine requirement is used in the gut. Stoll et al. (1998) have observed that about 60 % of orally supplied threonine is retained in the gut, which is consistent with the difference in requirement between Chen (1997) and the present results (Chapter 4).

When we tried to explain the difference between the enteral and parenteral threonine requirement, we noted that threonine is important in the maintenance of the mucus lining of the gastrointestinal tract. As outlined in Chapter 2, threonine is an integral constituent of intestinal mucin proteins. Mucin proteins provide the structural backbone of the mucus gels that provide lubrication and protection from pathogens. Without a well-formed mucus gel layer, the underlying mucosa is more susceptible to attack by bacteria such as *E. coli*. We therefore reasoned that mucin production would be impaired by restricting dietary intake of threonine. Therefore, an investigation into the relationship between THR and gut mucin production may further elucidate the difference in intravenous and oral THR requirement. The objective of the following experiment is to evaluate the effect of threonine intake and the route of supplementation on the quantity, location and type of gut mucins. Specifically, the effect of an inadequate supply of threonine on gut mucins

will be compared to an adequate supply of threonine. Also, the difference in gut mucins will be compared between threonine supplied orally and intravenously.

5.2 MATERIALS AND METHODS

5.2.1 ANIMALS AND SURGERY

Twenty-one male piglets (approximately 2-3 days of age), obtained from the minimal disease herd at the University of Alberta, were randomLy assigned to one of three THR treatments:

Group 1: DEFICIENT : 0.1 g/kg/d oral THR

Group 2: ADEQUATE : 0.6 g/kg/d oral THR

Group 3: SUPPLEMENTED: 0.1 g/kg/d oral THR + 0.5 g/kg/d intravenous THR

The piglets were anesthetized and catheters were placed in the stomach for intragastric feeding, in the left femoral vein for blood sampling, and in the left jugular vein for intravenous infusion of saline and/or threonine using procedures previously described (chapter 4).

5.2.2 DAILY MAINTENANCE

The basal diet composition was identical to that described in Chapter 4, aside from threonine (**Table 4.2.2.1**). The diet was kept isonigtrogenous by varying the concentration of the non-essential amino acid alanine. In the deficient group, threonine was supplied at a rate of 0.1 g/kg/d orally via the gastric catheter and sterile saline solution (1g/L) was administered via the jugular catheter at a rate of 2 mL/h. In the

adequate group, dietary threonine was supplied at a rate of 0.6 g/kg/d via the gastric catheter and a sterile saline solution was adminstered as in the deficient group. In the supplemented group, threonine was supplied via the gastric catheter at a rate of 0.1 g/kg/d and threonine (dissolved in saline) was administered into the jugular catheter at a rate of 0.5 g/kg/d to maintain an equal total threonine intake to that of the adequate group. The rate of infusion of the threonine-saline solution was also 2 mL/h. The piglets were maintained on their test diet for 8 days. On each day, the piglets were weighed and blood samples were collected for plasma threonine and urea analysis. The blood samples were drawn via the femoral catheter into heparinized syringes, transferred to 1.5 mL microcentrifuge tubes (Bio-Rad Laboratories, Mississauga, ON), and centrifuged for 3 min at 5000g (Biofuge, Heraeus Instruments, Canlab, Mississauga, ON). The plasma layer was removed and stored at -80°C for later analysis of plasma amino acids. Total urine (for nitrogen balance) was collected each day, weighed and 7 mL sample volumes were kept at 4°C for analysis. The diarrhea score of each piglet was also assessed, as described in section **5.2.3.** On the last day of the study, the piglet was anesthetized and the abdominal cavity opened. The distal end of the colon was tied off (with cotton thread) at the anus and the duodenum was tied off (at the pyloric sphincter and ligament of Treitz). The entire length of intestine was then removed and placed in ice-cold saline. The mesentery was removed and the length of the small intestine was measured. The duodenum was then separated and a small section (approx. 2 cm) was taken from the middle of the section into 10% formalin for histological analysis. The remaining duodenal section was then emptied of its lumenal contents by squeezing and the mucosa was

scraped off. The lumenal contents, mucosa, and remaining muscularis were weighed, frozen in liquid nitrogen and stored at -80 °C for further analysis. The ileum, taken as the last 10% of the length of the small intestine to the ileocaecal valve, was tied off and removed. The jejunum was separated into 3 equal lengths by tying off, in a distal-to-proximal direction, and removed. The colon was tied off at its mid-point, uncoiled, and separated as proximal and distal sections. Sampling of each section into formalin, scraping of mucosa, and freezing of resulting samples were all performed as for the duodenum.

5.2.3 INCIDENCE OF DIARRHEA

The incidence and severity of diarrhea was observed daily, according to the method of Ball and Aherne (1987). Severity was scored upon the visual inspection of the consistency of fecal material on a scale of 0-3: 0 represented no diarrhea; 1 a slight diarrhea; 2 a moderate diarrhea; and 3 a severe, highly fluid diarrhea. Diarrhea scores were taken for 6 of 7 piglets in each treatment group. Because diarrhea was not anticipated, scoring was incomplete for the first replicate of three piglets.

5.2.4 NITROGEN BALANCE

Nitrogen excretion from daily urine samples were determined by Kjeldahl analysis (Bradstreet 1965). Approximately 2 g of urine sample was pipetted into a 500 mL flask and a catalyst pack (containing 9.9 g K_2SO_4, 0.41 g HgO and 0.08 g $CuSO_4$) was added. 30 mL of concentrated H_2SO_4 was added and the samples were digested for 30 min. at

high temperature and then allowed to cool to room temperature. 300 mL of water was then added to the flask and mixed well. Approximately 1 g of Zn metal (20 mesh) was added, mixed and then 110 mL of 40% NaOH was added, swirled to mix, and heated. The resulting distillate (about 125 mL) was collected into 250 mL flasks containing 50 mL of 4% boric acid (containing bromocresol green and methyl red indicators). The ammonia was then titrated with 0.1 N H_2SO_4 to an end point of a light pink colour.

For conversion to protein content, the following calculation was used:

% Protein = % N x 6.25,

where % N = [(mL of H_2SO_4 - blank) x Mol. Wt. of N x Normality of H_2SO_4 x 100%] / mass of sample

5.2.5 PLASMA AMINO ACID ANALYSIS

Plasma amino acid concentrations in daily blood samples were determined by reverse-phase HPLC as previously described in chapter 4.

5.2.6 PLASMA UREA ANALYSIS

Plasma urea concentrations in daily blood samples were determined using a Blood Urea Nitrogen (BUN) Endpoint kit (Sigma Chemical Co., St. Louis, MO). 1 mL of BUN reagent (containing 4.0 mmol/L α-ketoglutarate, 0.25 mmol/L NADH, 16 000 U/L urease, 25 000 U/L glutamate dehydrogenase; pH 8.0) and 5 µL of plasma sample was added to a spectrophotometer cuvette, covered, inverted and incubated for 5 min. The

absorbance was read at 340 nm and subtracted from the absorbance of a reagent blank (using 5 μL of distilled, deionized H_2O). Conversion of absorbance to urea concentration was performed by using a standard curve of urea samples of known concentrations.

5.2.7 MUCIN ANALYSIS

5.2.7.1 ISOLATION OF CRUDE MUCIN

Crude mucin was isolated from mucosal scrapings according to modified procedures of Allen (1981) and Miller and Hoskins (1981). Mucosal scrapings were lyophilized and 0.5 g was weighed into a 50 mL polystyrene test tube. 25 mL NaCl (0.15M, with 0.02M sodium azide) was added and homogenized for 1 min at 4°C using a Polytron® homogenizer (Brinkman Instruments, Rexdale, ON). The samples were centrifuged immediately at 4°C for 30 min at 12,000 g. The upper aqueous layer was decanted into a second 50 mL polystyrene test tube and centrifuged again as before to remove insoluble material. 16 mL of aqueous supernatant was added to a pre-weighed 50 mL test tube and 24 mL of ice-cold ethanol was added to yield a final concentration of 60 % ethanol (v/v). The samples were allowed to precipitate overnight at -20 °C. The following day, the samples were centrifuged at 4 °C for 10 min at 1400 g. The supernatant was decanted, the pellet resolubilized in 16 mL NaCl (0.15M), cooled in an ice bath, and 24 mL of ice-cold ethanol was added. The samples were again allowed to precipitate overnight at -20 °C. The samples were then centrifuged again at 4 °C for 10 min at 1400 g and the procedure repeated until a clear supernatant was obtained. Finally, the precipitate was resolubilized in 1 mL of distilled, deionized H_2O and lyophilized.

5.2.7.2 MUCIN QUANTITATION BY CARBOHYDRATE ANALYSIS

Exactly 1.5 mL H_2SO_4 (12M) was added to 50 mg of isolated crude mucin and left to stand for 1 hr at room temperature. The solution was diluted to 3M with 4.5 mL distilled, deionized H_2O (ddH_2O) and hydrolyzed for 1 hr at 110 °C. 200 µL internal standard was added (N-methylglucamine for amino sugars, myo-inositol for neutral sugars, 10 mg/mL ddH_2O) and a 1mL aliquot of the acid hydrolysate was cooled in an ice bath, and made basic with 700 µL concentrated ammonium hydroxide. Of this, 100 µL was taken and 1 mL of sodium borohydride (30 mg/mL in anhydrous dimethylsulphoxide) was added. The Ring-opening reduction reaction was allowed to occur for 90 min at 40 °C. Excess sodium borohydrate was decomposed with 200 µL glacial acetic acid, 200 µL of 1-methlimidazole was added, then 2 mL of acetic anhydride. The acetylation reaction was allowed to occur for 10-15 min at room temperature. Excess acetic anhydride was decomposed with 5 mL distilled, deionized H_2O and cooled to room temperature. The alditol acetates were then extracted into 4mL dichloromethane by vigorous shaking and removal of the upper aqeuous layer. The acetates were washed twice with 4 mL distilled, deionized H_2O and evaporated to dryness under a stream of nitrogen (extra dry). The alditol acetates were redissolved in 1 mL dichloromethane and 0.5 µL was injected onto the gas chromatography (GC) column. The column used was a DB-17 fused silica capillary column (0.25 mm i.d. x 30 m), using He (1.5 mL/min) as the carrier gas. The injector temperature was set to rise from 60 °C to 270 °C at 150 °C /min and maintained for 20 min. The oven temperature was set to rise from 50 °C to 190 °C at 30 °C /min,

maintained for 3 min. and then up to 270 °C at 5 °C/min and maintained for 5 min. The flame ionization detector temperature was set at 270 °C.

5.2.8 ANALYSIS OF HISTOLOGICAL SAMPLES

Portions of the intestinal tract of approximately 2 cm in length were taken from the duodenum, mid jejunum, ileum and proximal colon. The samples were fixed in 10% neutral buffered formalin (Histoprep, Fisher Scientific, Pittsburgh, Pennsylvania) and routinely processed and embedded in Paraplast® Tissue Embedding Medium (Oxford Labware - Division of Sherwood Medical, St. Louis, MO). After fixation, longitudinal strips running parallel to the mesenteric border of intestine were trimmed from the anti-mesenteric border. The strips were placed in cassettes, and then into a Fisher Model 266 Histomatic Tissue Processor (Fisher Scientific, Pittsburgh, Pennsylvania) according to the following schedule of fixation (by formalin), dehydration (by ethanol), clearing (by xylene), and impregnation (by Paraplast®) (**Table 5.2.8.1**).

Table 5.2.8.1 Preparation of intestinal tissue samples for histological staining

Step	Reagent	Conc. (%)	Temp (C)	Vac.	Time (h)
1	Formalin	10	40	Y	2
2	Formalin	10	40	Y	3
3	Ethanol	70	40	Y	1
4	Ethanol	80	40	Y	1
5	Ethanol	95	40	Y	1
6	Ethanol	95	40	Y	1
7	Ethanol	100	40	Y	1
8	Ethanol	100	40	Y	1
9	Xylene		40	Y	1
10	Xylene		40	Y	1
11	Paraplast		60	Y	1
12	Paraplast		60	Y	1

The tissues were then embedded in melted Paraplast® using a Tissue Tek III Embedding Console (Miles Scientific, Division of Miles Laboratories, Inc., Naperville, IL). The paraffin blocks were then cooled on the Tissue Tek III cryo console and then sectioned on a Reichert Biocut 1130 microtome (Reichert-Jung Scientific Instruments, Belleville, ON). Serial 5 μm longitudinal sections of each tissue were cut and floated onto a Tissue Tek II 45°C waterbath, transferred to glass slides, and dried for 1 hr at 60°C. The slides were then stained with Gill's Hematoxylin (3 min) and eosin (15 sec.) (H&E), 1% Alcian blue (AB, pH 2.5, 1 hr) for the localization of carboxylated and/or sulphated acidic mucins; 1% Alcian blue (AB, pH 1.0, 1 hr) for the selective identification of sulphomucins (Carson 1990); a combination Alcian blue/periodic acid(5 min.)-Schiff base (15 min) reaction (Modified McManus Method, Mikel 1999) allowing unsubstituted alpha-glycol rich neutral mucins (pink) and acidic mucins (blue) to be differentiated. With this procedure, a redish-pink colour was obtained when only neutral mucins were present, a purple colour was obtained when both neutral and acidic mucins were present within the same goblet cell and a deep purple when acidic mucins were mainly present. Specific binding sites on the mucin protein for the stains are shown in **Fig. 2.3.2.2.** Finally, the slides were dipped three times in each of 90% ethanol, 100% ethanol, and xylene. A cover slide was then affixed to the slide and then subjected to morphometric analysis (Carson 1990).

Histomorphometric analyses of the stained sections were performed using a BH-2 binocular light microscope (Olympus, Japan) at 10x ocular magnification with a 10x

objective. The parameters measured were:

1. villus height (h, measured from the brush border of the most distal epithelial cell at the tip of the villus to the villus-crypt junction),
2. crypt depth (d, measured perpendicular to the muscularis mucosae from the crypt-villus junction to the basal lamina supporting the deepest epithelial cell in the base of the crypt),
3. villus width at at mid-villus height (mh)
4. villus width at the crypt-villus junction (vb).
5. villus height-to-crypt depth ratio, calculated as h/d,
6. villus-crypt cross sectional area, calculated as $vca = \pi*(1/2\ vb)^2$,
7. villus surface area was calculated as $vsa = (\pi*mh*h) + \pi*(1/2\ mh)^2$.

For all measurements and counts, only vertically oriented villi, crypts (small intestine) and colon gland ridges were selected. Longitudinal crypt and ridge sections were selected if elongated, straight, possessed an open lumenal margin, and had crypt or ridge base in contact with the muscularis mucosae.

Semiquantitative staining intensities were subjectively evaluated based upon a scale ranging from 0 (unreactive) to +++ (intensely stained). To ensure comparability between the different groups of animals, the sections from all experimental groups were stained in a single batch.

In addition, cells in the intestinal mucosa stained with AB 1.0, AB 2.5, or AB 2.5/PAS were counted in 10 well oriented crypt-villus units in each animal. An eyepiece graticule was oriented at the base of the crypt and the different cell types were counted in that

segment. The slide was then moved so that the graticule covered the adjacent segment and so on for sequential segments from the base of the crypt to the tip of the villus. Each segment was about 25 μm in length. The counts for 10 segments from the base were pooled and expressed as a mean due to variations in villus lengths and orientation.

5.2.9 STATISTICAL ANALYSIS

Statistical comparisons of measured parameters between threonine treatment groups were performed by Analysis of Variance (ANOVA), followed by the Least-Significant-Difference (LSD) multiple comparison test (SAS v.6.07, Cary, NC). Differences were considered to be significant at $p < 0.05$.

5.3 RESULTS

5.3.1 DAILY WEIGHT GAIN

During the course of the study, the piglets in the threonine-adequate and supplemented treatment groups were healthy and active while the piglets in the threonine-deficient group became listless after 1 to 2 days. The average initial weight of all the piglets was 1.84 kg (SD = 0.37), the average final weight was 2.73 kg (SD = 0.45), and the average daily gain in weight was 0.11 kg (SD = 0.03) (**Table 5.3.1.1**). Weights and weight changes were not significantly different among the different diet levels.

Table 5.3.1.1 Weight Changes in Piglets Fed Different Levels of Threonine

	Threonine Treatment Group				
Weight (kg)	Adequate	Deficient	Supplemented	Mean	SD_{pooled}
Initial	1.83	1.92	1.76	1.84	0.37
Final	2.82	2.81	2.53	2.73	0.45
Wt gain/d	0.12	0.11	0.10	0.11	0.03
n	7	7	7		

5.3.2 NITROGEN BALANCE

Nitrogen intake over the course of the study did not differ among the treatment groups ($p > 0.10$). Intakes were 2.00, 1.97 and 2.19 g N/kg/d (SD = 0.43) in the deficient, adequate and supplemented groups, respectively (**Table 5.3.2.1**). Baseline urinary nitrogen excretion at the onset of the test diet averaged 66.1, 69.0, and 67.7 g for treatment groups receiving 0.1 (deficient), 0.6 (adequate), and 0.1 + 0.5 i.v. (supplemented) g THR/kg/d, respectively (**Fig. 5.3.2.1**). These values were not significantly different between the three treatment groups. By day 2 of the study, the group receiving deficient THR had a mean urinary nitrogen excretion of 177.2g,

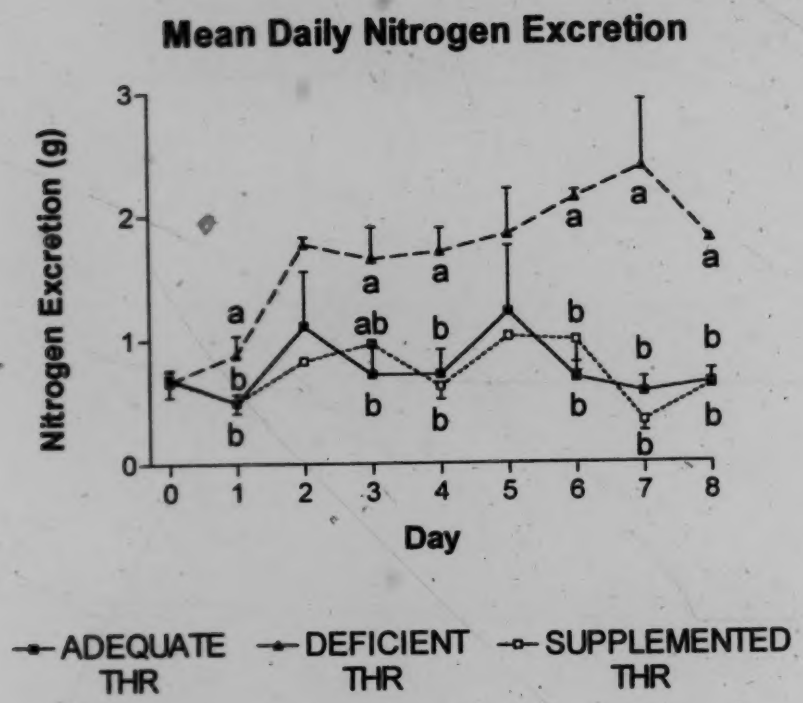


Figure 5.3.2.1 Mean daily nitrogen excretion in urine of piglets fed different levels of threonine (THR). Values are means ± SEM. Letters denote significant differences between THR treatment groups, $P < 0.05$.

compared to 111.5 g for the THR-adequate group and 83.2 g for the THR-supplemented group. From day 2 to day 8 of the study, urinary nitrogen excretion in the THR-deficient group remained stable, with a mean of 190.33 g. Nitrogen excretion in the THR-adequate and -supplemented groups also remained stable, at 81.15 and 77.16 g respectively. These latter two groups were not statistically different from each other while they were both significantly lower than the THR-deficient group ($p<0.05$). Nitrogen balance over days 2 to 8 were 1.4, 1.7 and 1.9 g/kg/d in the threonine deficient, adequate, and supplemented groups, respectively. Nitrogen retention was 70% in the deficient group and 84% in the threonine adequate and supplemented groups.

Table 5.3.2.1 Nitrogen Balance in Piglets Fed Different Levels of Threonine

	Threonine Treatment				
	Deficient	Adequate	Supplemented	SD_{pooled}	p
Nitrogen Intake (g/kg/d)	2.0	2.0	2.2	0.1	0.99
Nitrogen Output (g/kg/d)	0.7^a	0.3^b	0.3^b	0.14	0.0001
Nitrogen Balance (g/kg/d)	1.4	1.7	1.9	0.08	0.26
Nitrogen Retention (%)	70^b	84^a	84^a	7.1	<0.0001

^{a,b} denote significance in rows by LSD multiple comparison test ($p<0.05$).

5.3.3 PLASMA THREONINE LEVELS

Initial plasma threonine levels at the onset of the test diet were not significantly different from each other, at 408 umol/L (SD 99), 329 umol/L (SD 83) , and 324 umol/L (SD 76) in the deficient, adequate and supplemented groups, respectively (**Fig. 5.3.3.1**). In the deficient group, plasma threonine decreased at day 1 and remained relatively constant over days 1 to 8, at 53 umol/L (SD 16). Similarly, the threonine concentration in the adequate group also decreased by day 1 and remained constant from day 1 to 8, at an average of 181umol/L (SD 32). In the threonine supplemented group however, plasma threonine increased between days 0 to 4, and then remained relatively similar between days 4 and 8 at approximately 572 umol/L (SD 33).

During days 1 to 8, plasma threonine concentrations between the adequate and deficient groups were not significantly different from each other but were both significantly lower than the threonine supplemented group.

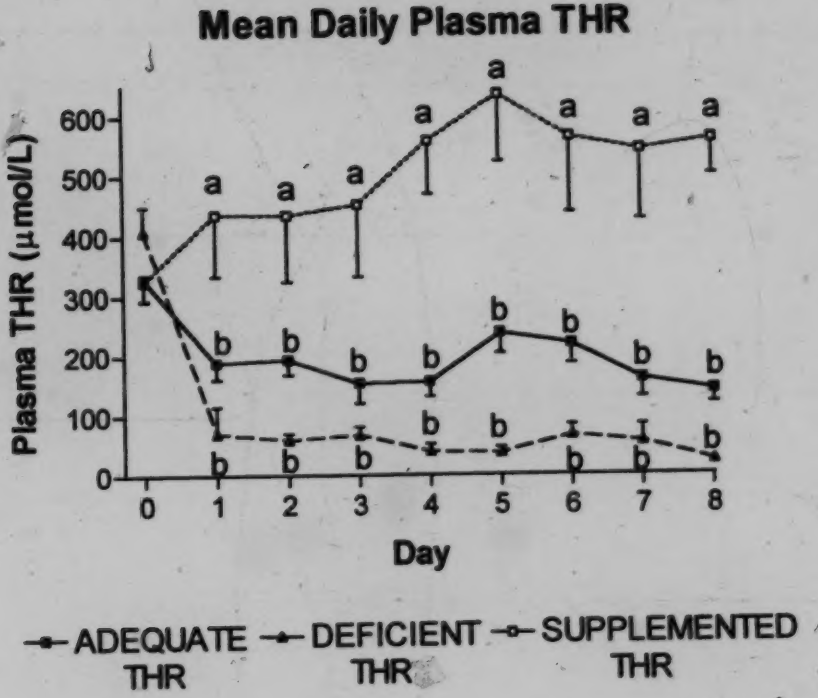


Figure 5.3.3.1 Mean daily plasma threonine (THR) concentration in piglets fed different levels of THR. Values are means ± SEM. Letters denote significant differences between THR treatment groups, $P < 0.05$.

5.3.4 PLASMA UREA

Plasma urea levels at the beginning of the study were not different at 1.26, 1.51, and 1.36 mM respectively, for treatment groups receiving deficient (0.1 g/kg/d), adequate (0.6 g/kg/d), and supplemented (0.1 + 0.5 i.v. g/kg/d) amounts of THR (**Fig. 5.3.4.1**). By day 2, the plasma urea levels in piglets receiving deficient THR had risen significantly to 8.40 mM ($p < 0.05$), compared to the levels in the THR-adequate and THR-supplemented groups which were 1.83 and 1.97 mM respectively. Between day 2 and the end of the study, plasma urea in the THR-deficient group remained relatively constant, with a mean of 7.89 mM (SD 2.2). During this same period, plasma urea also remained constant in the other two treatment groups, with means of 1.98 (SD 1.2) and 2.53 mM (SD 1.1) respectively, for the THR-adequate and -supplemented groups. Although the plasma urea levels did not differ significantly between these two groups, each was significantly lower than the THR-deficient group ($p<0.05$).

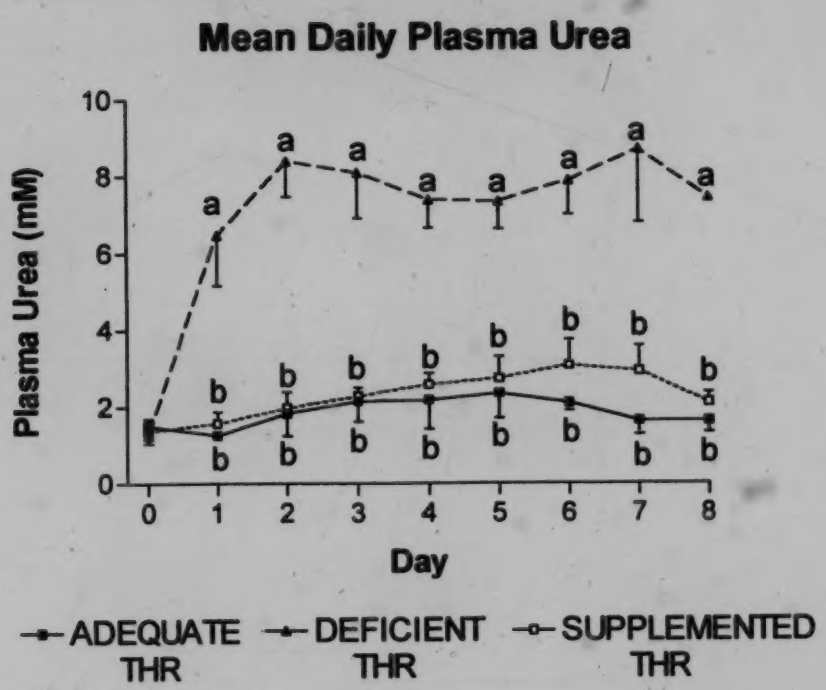


Figure 5.3.4.1 Mean daily plasma urea concentration in piglets fed different levels of threonine (THR). Values are means ± SEM. Letters denote significant differences between THR treatment groups, $P < 0.05$.

5.3.5 INCIDENCE OF DIARRHEA

A total of 6 piglets in the THR-deficient group exhibited diarrhea on 35 out of a possible 60 pig days, with an average severity of 2.82 over the diarrhea days (**Table 5.3.5.1**). Mean daily scores for all pigs in this treatment group over the entire study period was 1.6. Severe diarrhea was observed by the 4th day of the study and the change to severe diarrhea was abrupt.

In the THR-adequate group, only 1 piglet exhibited diarrhea over a total of 2 days, with an average severity of 1.5 and an overall mean daily score of 0.05 for all piglets within the treatment group.

In the THR-supplemented group, 2 piglets were observed to have slight and moderate diarrhea, over 2 and 7 days, respectively. The average severity of diarrhea over the 9 diarrhea days was 1.3, and the mean daily score for all piglets within the treatment group was 0.2.

Although 2 piglets in the THR-deficient group died prematurely on the final day of the study, it is unlikely that the cause of the diarrhea was bacterial in nature. Growth (measured as daily weight increase) was similar to piglets not showing evidence of diarrhea. Post mortem evaluation by a veterinary pathologist revealed no evidence of clinical intestinal disease. The cause of diarrhea in the piglets was likely due to threonine deficiency as severe diarrhea was observed only in the THR-deficient group and came about suddenly.

Table 5.3.5.1 Incidence of Diarrhea

Treatment	n	Mean Daily Score[1] (0-3)	# Pigs w/ Diarrhea	# Diarrhea Days	Avg. Severity[2] (0-3)
Deficient	6	1.63[a]	6	35	2.82[a]
Adequate	6	0.05[b]	1	2	1.50[b]
Supplemented	6	0.2[b]	2	9	2.21[b]
SD_{pooled}		0.83			0.83

[1] Mean Daily Score: average of the sum of daily scores / # days of study
[2] Average Severity: average daily score on days where diarrhea was present
[a,b] denote significance by LSD multiple comparison test ($p<0.05$)

5.3.6 TISSUE WEIGHTS

The data for gut length, gut weight, and mucosal weight are described in **Table 5.3.6.1** and **Table 5.3.6.2**. In the duodenum, all parameters measured were not significantly different among the different treatment groups, except for mucosal weight. The wet mucosal weight in the threonine-adequate group was 1.1 g and significantly higher than in the deficient group (0.63 g) ($p<0.05$). The wet mucosal weight of the supplemented group was not different from either of the other groups (0.91 g) ($p>0.05$).

In the proximal jejunum, the mucosal weights were also significantly different ($p<0.05$) among treatment groups, again with the adequate group containing the highest value, the deficient group containing the lowest, and the supplemented group in between the other two groups. Also in the proximal jejunum, the length of the jejunum itself, when normalized by the final weight of the piglet, was found to be different between treatment groups, in the same pattern of significance as with the mucosal weight.

In the mid and distal jejunum, wet tissue weight was significantly greater in the deficient group than in the supplemented group while the wet tissue weight in the adequate group was intermediate in value and not different from either of the other two groups. Also in the mid and distal jejunum, the wet tissue weight, when normalized against final body weight, was significantly greater in the deficient group than in the other groups which were not different from one another. In the distal jejunum alone the weight of the luminal contents were highest in the adequate group and lowest in the deficient group while the supplemented group was intermediate and not different from the others. In the ileum, there were no significant differences found among any of the treatment groups in the parameters measured.

Table 5.3.6.1 Small Intestinal Parameters

Section	Parameter	Treatment			SD_{pooled}
		Adequate	Deficient	Supplemented	
Duodenum	length (cm)	12.1	10.6	11.9	2.1
	length/final wt.* (cm/kg)	4.4	3.7	4.7	1.1
	luminal wt. (g)	0.70	0.73	0.43	0.50
	luminal wt./length (g/cm)	0.06	0.07	0.03	0.05
	mucosal wt. (g)	1.1[a]	0.63[b]	0.91[ab]	0.35
	mucosal wt./length (g/cm)	0.09	0.06	0.08	0.03
	muscularis wt. (g)	0.96	1.00	1.00	0.35
	muscularis wt./length (g/cm)	0.08	0.09	0.09	0.03
Proximal Jejunum	length (cm)	191.0	142.6	173.6	42.8
	length/final wt.* (cm/kg)	69.6[a]	51.0[b]	68.5[ab]	16.0
	luminal wt. (g)	9.2	6.2	6.5	4.2
	luminal wt./length (g/cm)	0.05	0.04	0.04	0.02
	mucosal wt. (g)	19.4[a]	11.5[b]	17.6[ab]	6.3
	mucosal wt./length (g/cm)	0.10	0.08	0.10	0.02
	muscularis wt. (g)	12.4	11.2	11.0	3.0
	muscularis wt./length (g/cm)	0.07	0.08	0.06	0.02
Mid Jejunum	length (cm)	174.7	150.6	158.9	27.0
	length/final wt.* (cm/kg)	63.3	52.8	63.9	10.0
	luminal wt. (g)	6.6	3.8	3.6	3.4
	luminal wt./length (g/cm)	0.04	0.03	0.02	0.02
	mucosal wt. (g)	18.6	14.2	16.3	4.2
	mucosal wt./length (g/cm)	0.11	0.10	0.10	0.03
	muscularis wt. (g)	11.0[ab]	12.6[a]	9.2[b]	2.6
	muscularis wt./length (g/cm)	0.06[b]	0.09[a]	0.06[b]	0.02
Distal Jejunum	length (cm)	174.9	150.2	158.9	26.9
	length/final wt.* (cm/kg)	63.4	52.7	63.9	26.9
	luminal wt. (g)	5.0[a]	2.2[b]	3.9[ab]	2.0
	luminal wt./length (g/cm)	0.03	0.02	0.02	0.01
	mucosal wt. (g)	18.7	14.8	16.8	4.3
	mucosal wt./length (g/cm)	0.11	0.10	0.11	0.03
	muscularis wt. (g)	11.6[ab]	13.8[a]	9.9[b]	2.5
	muscularis wt./length (g/cm)	0.07[b]	0.10[a]	0.06[b]	0.03
Ileum	length (cm)	58.1	55.0	54.0	7.5
	length/final wt.* (cm/kg)	21.1	19.3	21.5	2.2
	luminal wt. (g)	1.3	0.48	1.2	0.8
	luminal wt./length (g/cm)	0.02	0.01	0.02	0.01
	mucosal wt. (g)	6.4	4.9	6.0	1.7

mucosal wt./length (g/cm)	0.11	0.10	0.11	0.03
muscularis wt. (g)	5.2	5.6	4.8	1.0
muscularis wt./length (g/cm)	0.10	0.10	0.9	0.02
Total Small Intestine length (cm)	583	552	539	71
Total Small Intestine length/ final wt.* (cm/kg)	211	194	215	21

[a, b,c] denotes significance at $p < 0.05$ by LSD multiple comparison test
*Final weight of piglet

Table 5.3.6.2 Large Intestinal Parameters

		Treatment			
Section	Parameter	Adequate	Deficient	Supplemented	SD_{pooled}
Proximal Colon	length (cm)	57.0	46.7	55.7	10.1
	length/final wt.* (cm/kg)	20.9^{ab}	16.5^{b}	22.5^{a}	4.8
	luminal wt. (g)	7.4^{a}	1.2^{b}	6.6^{a}	3.8
	luminal wt./length (g/cm)	0.13^{a}	0.02^{b}	0.12^{a}	0.07
	mucosal wt. (g)	3.6^{a}	1.8^{b}	3.2^{a}	1.1
	mucosal wt./length (g/cm)	0.06^{a}	0.04^{b}	0.06^{a}	0.02
	muscularis wt. (g)	11.6^{a}	7.3^{b}	10.3^{ab}	3.2
	muscularis wt./length (g/cm)	0.21	0.16	0.19	0.06
Distal Colon	length (cm)	63.1	50.5	64.0	11.9
	length/final wt.* (cm/kg)	23.1^{ab}	17.7^{b}	25.6^{a}	5.3
	luminal wt. (g)	6.6^{a}	0.6^{b}	4.3^{a}	3.6
	luminal wt./length (g/cm)	0.10^{a}	0.01^{b}	0.07^{a}	0.05
	mucosal wt. (g)	3.8^{a}	1.8^{c}	2.9^{b}	1.1
	mucosal wt./length (g/cm)	0.06^{a}	0.04^{b}	0.05^{ab}	0.02
	muscularis wt. (g)	9.1	6.3	8.7	2.6
	muscularis wt./length (g/cm)	0.14	0.12	0.14	0.04
Total Large Intestine length (cm)		120.1	97.2	119.7	20.3
Total Large Intestine length/ final wt.* (cm/kg)		44.0^{ab}	34.3^{b}	48.1^{a}	9.6

[a, b,c] denotes significance at $p < 0.05$ by LSD multiple comparison test
*Final weight of piglet

In the large intestine, separated into the proximal and distal colon, piglets in the adequate and supplemented group exhibited significantly greater weight of luminal contents, luminal content/length of section sampled, mucosal weight and mucosal weight/length of section sampled. In each case, the values from piglets in the supplemented group were not different from those from the adequate group. In addition, the length of the proximal colon when normalized against final weight was greatest in the supplemented group, lowest in the deficient group and intermediate in the adequate group. The wet tissue weight in the proximal colon was highest in the adequate group and lowest in the deficient group with the supplemented group in between and not different from the others.

The general trend from the gross parameters measured showed higher values in the adequate and supplemented group compared to the deficient group, although many of these differences were not found to be significant.

5.3.7 HISTOCHEMISTRY

5.3.7.1 NUMBER OF GOBLET CELLS BY STAINING METHOD

5.3.7.1.1 STAINING BY PAS/ALCIAN BLUE at pH 2.5

In almost all sections of the gut under all treatments, stained goblet cells were predominantly purple, indicating a homogeneous mixture of both neutral and acidic mucins. The exception was the colon of the THR-deficient group, in which stained cells were mainly dark blue in colour, indicating acidic mucins. There was also a greater abundance of blue-stained goblet cells in the colon than in any of the small intestinal

sections.

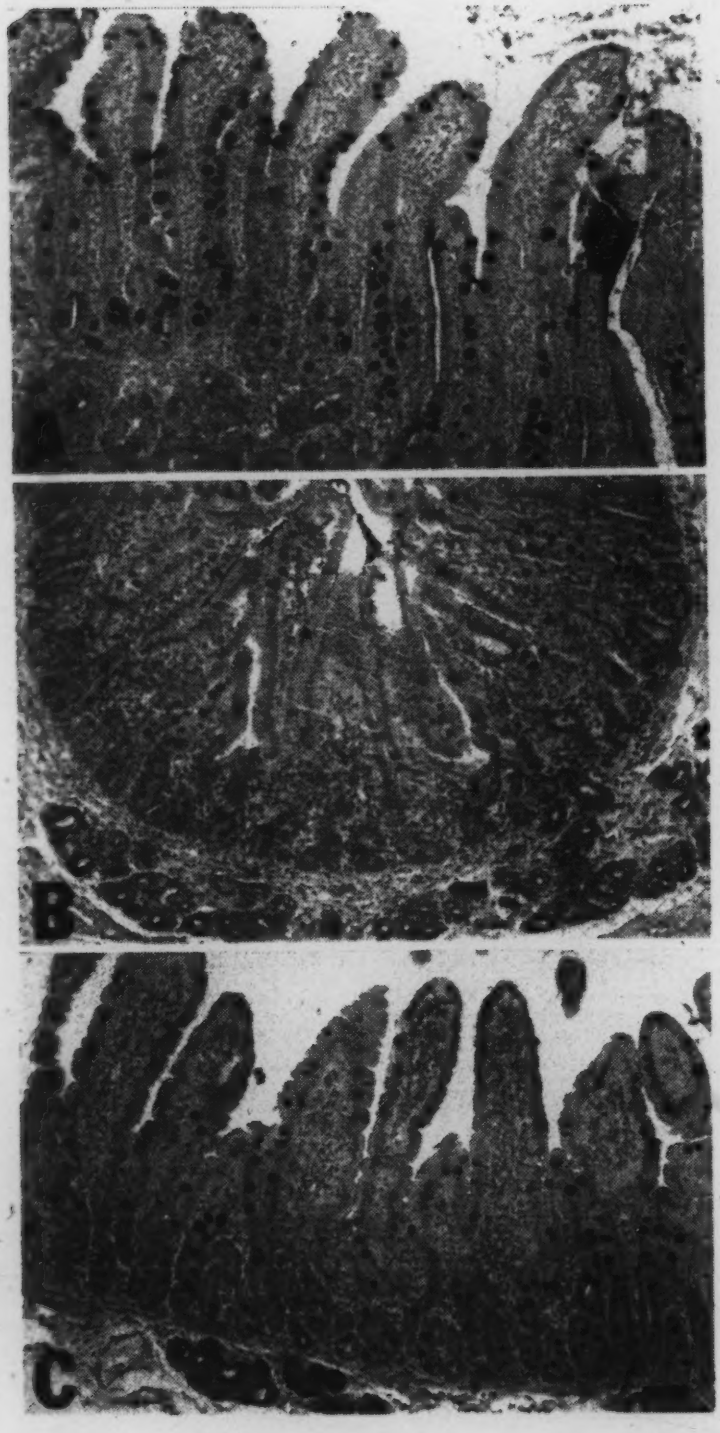
In the duodenum, there was little staining on surface areas in all three treatment groups. A significantly ($p<0.05$) greater number of mucin-containing goblet cells were seen in the duodenum of the THR-adequate group (18.8) than in both the THR-deficient (15.2) and THR-supplemented (14.9) groups (**Table 5.3.7.1, Fig. 5.3.7.1 A, B, C**). Furthermore, these cells were predominantly stained red (neutral mucins) and purple (mixed neutral and acidic mucins) in the zones of proliferation and cell transit. There was limited staining of blue (acidic mucins) and these were located within the deep crypts.

Table 5.3.7.1 Goblet Cell Summary: PAS/AB 2.5

			PAS/AB 2.5				
Tissue	Treatment	Total Cells	RR	P	DB	R/P	R/B
Duodenum	Adequate	18.8^{a}	5	7.6	2	0	4.3^{a}
	Deficient	15.2^{b}	4.5	7.4	0.1	0	3.1^{ab}
	Supplem.	14.9^{b}	4.5	8.6	1.3	0	0.4^{b}
	SD_{pooled}	2.7	1.8	3.5	2.1	0	1.9
Mid Jejunum	Adequate	16.7	5.7	10	0.5	0	0.1^{b}
	Deficient	15.9	4.9	9.2	0.2	0	1.6^{a}
	Supplem.	20.8	7.3	13	0.6	0	0.4^{b}
	SD_{pooled}	5.5	2.2	4.4	0.4	0	0.5
Ileum	Adequate	20.9	6^{ab}	14	1.4	0^{b}	0.7
	Deficient	22.6	5.6^{b}	13.8	0.9	0^{b}	2.3
	Supplem.	27.5	9.1^{a}	14	2.5	1.4^{a}	0.2
	SD_{pooled}	5.9	2.0	6.6	1.4	0.6	1.4
Proximal Colon	Adequate	30.0	3.9	13	6^{b}	5.3^{a}	1.4^{b}
	Deficient	33.1	4.3	5.8	17^{a}	0.2^{b}	5.3^{a}
	Supplem.	22.9	3.1	11	4.5^{b}	3.4^{ab}	0.5^{b}
	SD_{pooled}	8.3	1.6	7.2	3.4	2.6	1.6

$^{a, b,c}$ denotes columnwise significance at $p < 0.05$ by LSD multiple comparison test
RR-bright rose red (PAS staining of neutral mucins)
P-purple (homogeneous mixture of acidic and neutral mucins)
DB-deep blue (Alcian blue staining of acidic mucins)
R/P-red staining with discrete areas of purple (dual staining)
R/B-red staining with discrete areas of blue (dual staining)

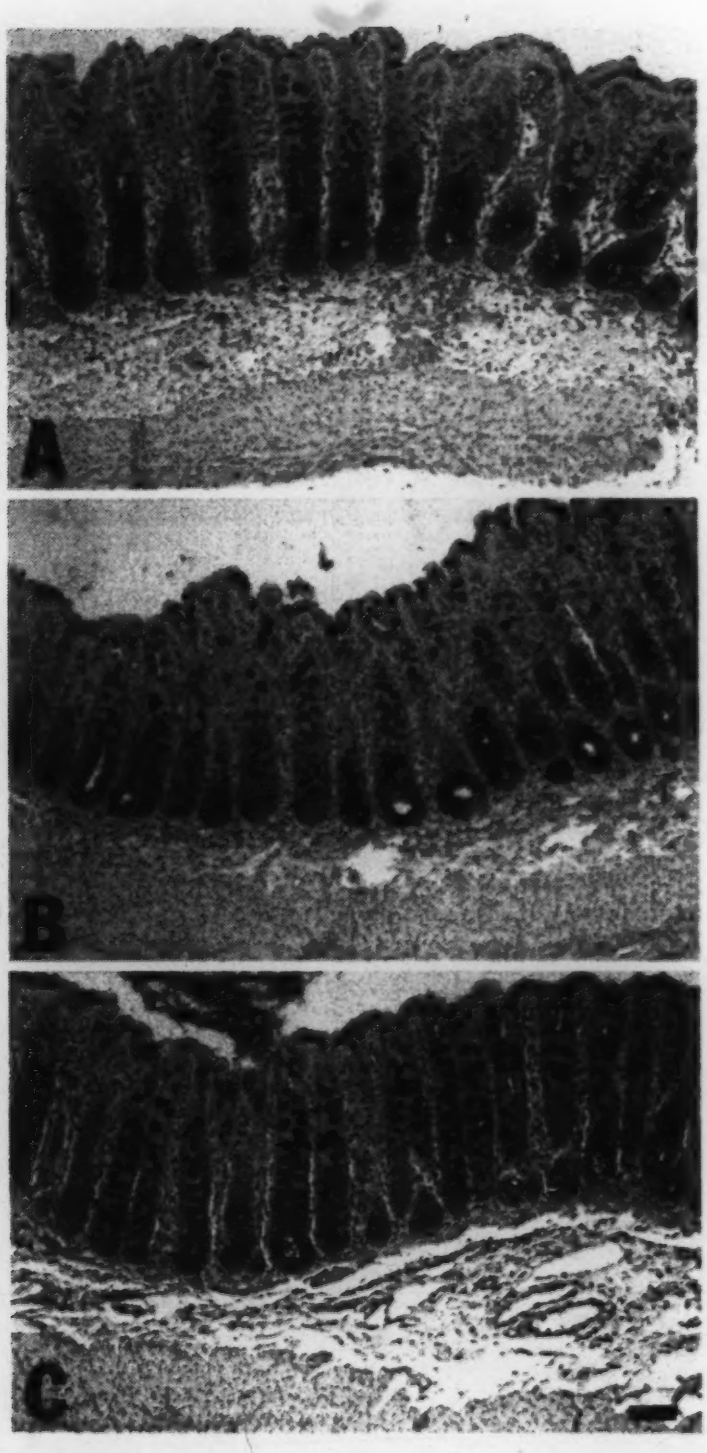
Figure 5.3.7.1 Light micrographs of duodenal mucosa in piglets fed threonine (THR). A: THR-adequate diet, fed intragastrically (IG). B: THR-deficient diet, fed IG. C: THR-supplemented diet, fed a deficient THR diet IG with THR supplementation given intravenously (IV). Stains are hematoxylin and eosin (A-C), goblet cell periodic acid-Schiff (PAS) staining of neutral mucins red/pink and goblet cell Alcian Blue (AB, pH 2.5) staining of acidic mucins blue (A-C). Goblet cells in A are more numerous and larger than in both B and C. Neutral and mixed neutral/acidic mucins (purple) predominate in the mid-villus while acidic mucins predominate in the deep crypts. Scale bar in C = 100 μm (A-C).


B
C

In the mid-jejunum and ileum, the THR-deficient group showed the fewest number (15.9) and the THR-supplemented group showed the greatest number (20.8) of mucin-containing goblet cells although these differences were not found to be significantly different. A similar pattern of colouration was observed in the jejunum and ileum as in the duodenum.

In the proximal colon, cells of the THR-deficient pigs contained the greatest number of total stained cells (33.1) **(Fig. 5.3.7.2 B)** and the cells of the THR-supplemented pigs contained the least number of stained cells (22.9) **(Fig. 5.3.7.2 C)**, although the differences between the groups were not found to be statistically different. Red and pink staining cells were more numerous in THR-adequate pigs **(Fig. 5.3.7.2 A)**. In contrast to the sections of the small intestine and to the other areas of the colonic villus, the deep crypts of the colon exhibited a greater amount of blue-staining goblet cells (indicating acidic mucins) than red staining goblet cells (indicating neutral mucins). As mentioned above, these blue-staining cells were found to be more prominant in number than the purple-staining cells that dominated in all other sections of the colon (surface, upper crypt, mid crypt) and small intestine (villus, crypt).

Figure 5.3.7.2 Light micrographs of proximal colonic mucosa in piglets fed threonine (THR). A: THR-adequate diet, fed intragastrically (IG). B: THR-deficient diet, fed IG. C: THR-supplemented diet, fed a deficient THR diet IG with THR supplementation given intravenously (IV). Stains are hematoxylin and eosin (A-C), goblet cell periodic acid-Schiff (PAS) staining of neutral mucins red/pink and goblet cell Alcian Blue (AB, pH 2.5) staining of acidic mucins blue (A-C). Staining intensity in A is greater than in both B and C. Neutral and mixed neutral/acidic mucins (purple) predominate in the mid-villus while acidic mucins predominate in the deep crypts. Acidic mucins are more numerous in B than in A. Scale bar in C = 100 μm (A-C).


A
B
C

5.3.7.1.2 STAINING BY ALCIAN BLUE at pH 1.0

Treatment by Alcian Blue staining at pH 1.0, sulphated acidic mucins were blue in colour. In the duodenum of the THR-deficient group, the most striking observation was the paucity of mucin-producing goblet cells (7.1) **(Table 5.3.7.2, (Fig. 5.3.7.3 B)**. In the THR-adequate group, more than twice as many ($p<0.05$) mucin-producing cells could be seen (16.3), with the cells of the villus-crypt junction more deeply stained than in the crypt **(Fig. 5.3.7.3 A)**. This indicates a greater amount of mucin in the cells of this latter region. This distribution of staining was opposite to that achieved when gut sections were treated by Alcian Blue at pH 2.5 (staining for sialylated mucins) and is in agreement with other investigators' results pertaining to the gut in pigs **(Chapter 2)**. In the duodenum of THR-supplemented piglets, a numerically greater number of mucin-producing cells were seen (13.5) than in the THR-deficient group (7.1) but less than that found in the THR-adequate group (16.3) **(Fig. 5.3.7.3 C)**. The number of goblet cells in this treatment group did not differ significantly from either of the other two groups.

In the mid jejunum and ileum, the same trends were observed as seen in the duodenum although the differences between treatment groups were not found to be significant.

In the proximal colon, The number of mucin-producing goblet cells were found to be similar between the THR-adequate and THR-deficient groups (23.8, 23.7 respectively) **(Fig. 5.3.7.4 A, B)**. The number of such cells in the THR-supplemented group was slightly greater (28.9) but not found to be different than either of the other groups **(Fig. 5.3.7.3 C)**. Also, stained cells were seen to be located in the deep and mid crypts in the THR-deficient group while stained cells in THR-adequate and THR-supplemented groups

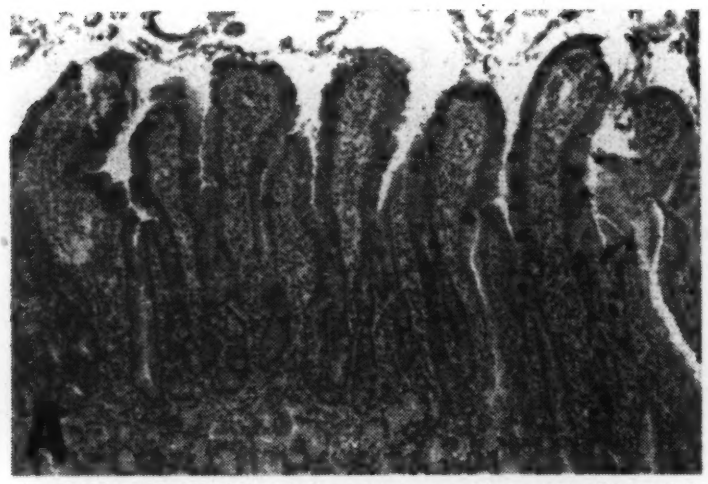
were seen throughout the length of the villi.

Table 5.3.7.2 Goblet Cell Summary: AB 2.5 and AB 1.0

		Total Cells	
Tissue	Treatment	AB 2.5	AB 1.0
Duodenum	Adequate	17.6[a]	16.3[a]
	Deficient	5.5[c]	7.1[b]
	Supplem.	12.6[b]	13.5[ab]
	SD_{pooled}	4.4	4.4
Mid	Adequate	9.1	7.6
Jejunum	Deficient	7	6
	Supplem.	8.7	7.5
	SD_{pooled}	1.7	2.7
Ileum	Adequate	30.1[a]	24.6[a]
	Deficient	13.9[b]	13.3[b]
	Supplem.	19.9[ab]	20.7[ab]
	SD_{pooled}	7.8	6.5
Proximal	Adequate	30	23.8
Colon	Deficient	29.9	23.7
	Supplem.	24.9	28.9
	SD_{pooled}	6.9	6.8

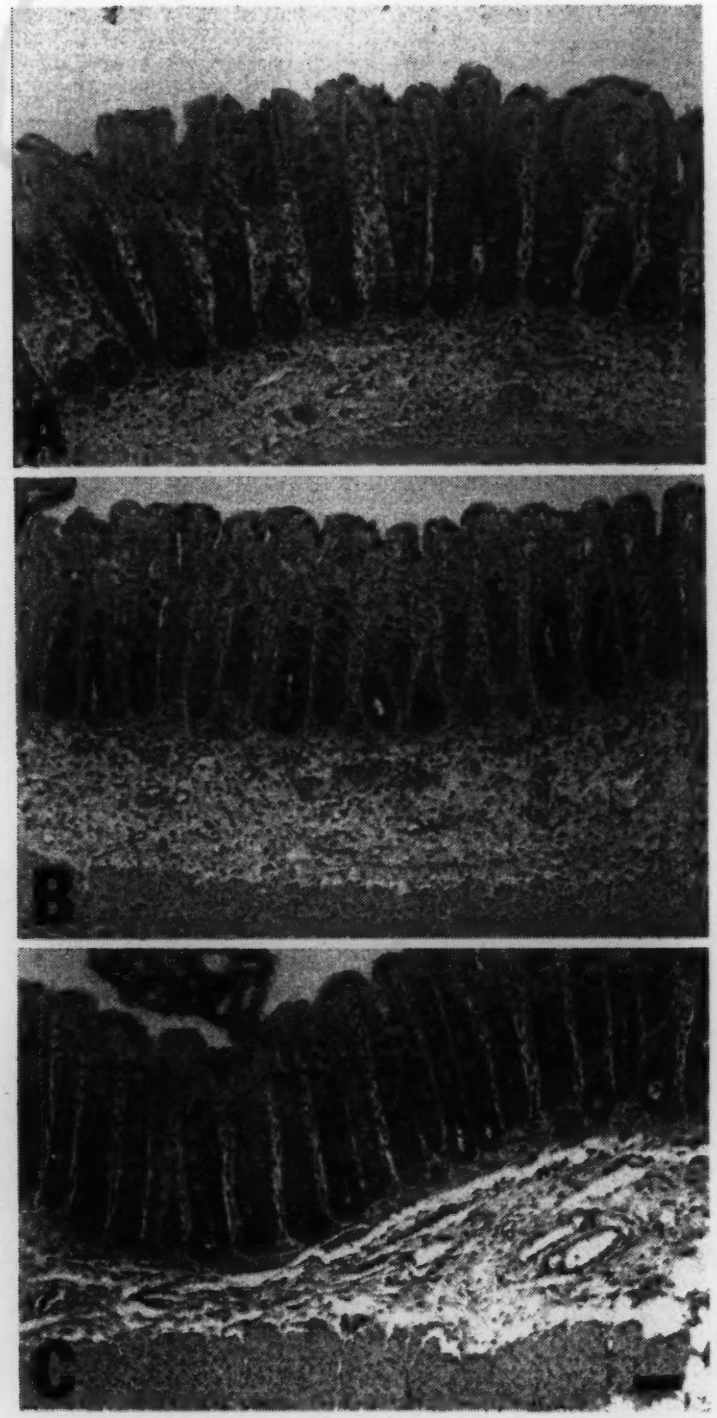
[a, b,c] denote significance at $p < 0.05$ by LSD multiple comparison test

Figure 5.3.7.3 Light micrographs of duodenal mucosa in piglets fed threonine (THR). A: THR-adequate diet, fed intragastrically (IG). B: THR-deficient diet, fed IG. C: THR-supplemented diet, fed a deficient THR diet IG with THR supplementation given intravenously (IV). Stains are hematoxylin and eosin (A-C), and goblet cell Alcian Blue (AB, pH 1.0) staining of sialylated acidic mucins blue (A-C). Goblet cells in A are more numerous and larger than in B while goblet cells in C are as large but not as numerous as in A. Villi in B are less well formed and exhibit lower surface area than in A. Scale bar in C = 100 μm (A-C).


A

B

Figure 5.3.7.4 Light micrographs of proximal colonic mucosa in piglets fed threonine (THR). A: THR-adequate diet, fed intragastrically (IG). B: THR-deficient diet, fed IG. C: THR-supplemented diet, fed a deficient THR diet IG with THR supplementation given intravenously (IV). Stains are hematoxylin and eosin (A-C), and goblet cell Alcian Blue (AB, pH 1.0) staining of sialylated acidic mucins blue (A-C). Goblet cells are equally numerous in all three groups and staining intensity is greatest in B. Sialylated mucins predominate in the crypt and mid villus in B while locating along the entire length of the villus in A and C. Scale bar in C = 100 μm (A-C).


A
B
C

5.3.7.1.3 STAINING BY ALCIAN BLUE AT pH 2.5

In the duodenum of the THR-deficient group, a paucity of mucin-producing goblet cells were observed (5.5) (**Table 5.3.7.2**). In the THR-adequate group, there was significantly ($p<0.05$) more sialylated mucins (17.6). The number of goblet cells seen in the THR-supplemented group (12.6) was also significantly different ($p<0.05$) from both of the other two groups. In all groups, the pattern of staining was similar, with a decrease in staining intensity from deep crypt to villus tip.

In the mid jejunum and ileum, the same trends were seen as in the duodenum, with the THR-adequate containing the greatest number of mucin-producing cells, the THR-deficient group the least, and the THR-supplemented group containing an intermediate number. The differences between the groups were not significant however.

In the colon of the THR-deficient group, the stained cells were clustered in the deep crypts and in the zone of proliferation while the cells of the THR-adequate and THR-supplemented groups were distributed along the entire length of the villus. Additionally, the cells in the THR-adequate group were observed to be greater in size than the cells from the THR-deficient and THR-supplemented groups. Goblet cell number were similar between the THR-adequate and THR-deficient groups (30 and 29.9 respectively), while there was a non-significantly smaller number found in the THR-supplemented group (24.9).

5.3.7.2 STAINING INTENSITY

The staining intensity of goblet cells was found to be significantly greater in the ileum

and proximal colon of the THR-supplemented group under staining by AB 1.0. **(Table 5.3.7.3)**. In the mid jejunum, the staining intensity followed the same trends although not significantly. Regionally, the staining intensity was found to be greater in the crypt than in the villus for THR-adequate and THR-deficient groups. The one exception was the duodenum of the THR-adequate group. For all sections of the small intestine, villus and crypt intensities in the THR-deficient group were similar. In the proximal colon, staining intensity was greater in the lower crypt than in the upper crypt or surface.

In the duodenum, mid jejunum and ileum, the staining intensity by AB 2.5 was significantly lower in the THR-deficient group than in the THR-adequate and THR-supplemented groups, which were not different from one another **(Table 5.3.7.3)**. In the colon, staining intensity was significantly greater in the THR-deficient group than in the THR-supplemented group, with the THR-adequate group not different from either of the other two groups. Regionally, staining intensity in the small intestine was again greater in the crypt than in the villus (including the duodenum of the THR-adequate group), and intensities were again found to be equal between villus and crypt in THR-deficient groups. Staining intensity of the proximal colon was also found to be greatest in the lower crypt.

In goblet cells stained by PAS/AB 2.5, the staining intensity in the duodenum, mid jejunum and proximal colon was lower in the THR-deficient group than the other two treatment groups, although significance was only found in the mid jejunum. Regionally, all sections of the small intestine under all treatment groups were found to be intensely stained red (neutral mucins) and purple (mixed mucins). The intensity of blue stained (acidic mucins) cells was equal among treatment groups in the ileum, and lower in the

THR-deficient group in the duodenum and mid jejunum. In the proximal colon, the intensity of staining of neutral, acidic and mixed mucins were equally high in the THR-adequate group. In the THR-supplemented group, staining intensities were lower but equal among neutral, acidic and mixed mucins. In the THR-deficient group, staining intensity was found to be still lower, with mixed mucins staining less intensely than neutral and acidic mucins. Comparing between treatment groups, staining intensities were found to be lower in the THR-deficient group than in either of the other two treament groups for all types of mucins (neutral, acidic, mixed neutral and acidic).

Table 5.3.7.3 Average Staining Intensity

Tissue	Treatment	*n*	AB 1.0	*n*	AB 2.5	*n*	PAS/AB 2.5
Duodenum	Adequate	6	1.5	7	2.0^{a}	5	3.0
	Deficient	3	1.7	4	1.0^{b}	3	2.7
	Supplem.	6	2.2	6	2.0^{a}	5	2.6
	SD_{pooled}		1.3		0.9		1.3
Mid Jejunum	Adequate	7	1.2	7	1.3^{a}	6	2.7^{xy}
	Deficient	5	1.0	5	1.0^{b}	5	2.3^{y}
	Supplem.	5	1.3	6	1.3^{a}	4	3.0^{x}
	SD_{pooled}		0.70		0.4		1.3
Ileum	Adequate	3	1.2^{b}	6	1.8^{a}	4	3.0
	Deficient	5	1.0^{b}	5	1.0^{b}	5	3.0
	Supplem.	6	1.5^{a}	7	1.6^{a}	5	3.0
	SD_{pooled}		0.3		0.5		0
Proximal Colon	Adequate	6	1.2^{b}	6	1.8^{ab}	4	3.0
	Deficient	5	1.5^{b}	4	2.2^{a}	5	2.3
	Supplem.	5	2.3^{a}	6	1.6^{b}	7	2.7
	SD_{pooled}		1.2		1.2		1.5

$^{a, b,c}$ denotes significance at $p < 0.05$ by Tukey's multiple comparison test
$^{x, y, z}$ denotes significance at $p < 0.05$ by LSD multiple comparison test

5.3.7.3 AVERAGE STAINING INTENSITY AND NUMBER OF GOBLET CELLS

As an additional means of understanding the effects of treatment on mucin production, the product of staining intensity and the number of goblet cells observed was calculated to give an estimate of total mucin content. These results are presented in **Table 5.3.7.4.** In goblet cells stained by AB 1.0, this product was lowest in the THR-deficient group than in the other groups, although the differences were not significant. In the proximal colon, the THR-supplemented group was found to have significantly greater mucin content than the other two groups.

Table 5.3.7.4 Average Staining Intensity x #Goblet Cells

Tissue	Treatment	AB 1.0	AB 2.5	PAS/AB 2.5
Duodenum	Adequate	27	44[a]	39
	Deficient	12	6[b]	41
	Supplem.	31	25[ab]	39
	SD_{pooled}	14.9	12.3	13.6
Mid Jejunum	Adequate	10	10	46
	Deficient	5	7	38
	Supplem.	10	12	63
	SD_{pooled}	6.1	4.6	18.2
Ileum	Adequate	30	63[a]	79
	Deficient	13	14[b]	68
	Supplem.	31	31[ab]	82
	SD_{pooled}	11.1	18.3	17.7
Proximal Colon	Adequate	31[b]	56	90
	Deficient	34[b]	60	72
	Supplem.	63[a]	40	60
	SD_{pooled}	10.1	17.2	27.2

[a, b,c] denotes significance at $p < 0.05$ by LSD multiple comparison test

In cells stained by AB 2.5, the sections of the small intestine showed the same trends as staining by AB 1.0. The duodenum and ileum of the THR-adequate group was found to be significantly greater than the deficient group, with the supplemented group intermediate and not different from the other two groups. In the proximal colon, the product of staining intensity and number of goblet cells was highest in the THR-deficient group, although the differences between the three treatment groups were not significant.

In cells stained by PAS/AB 2.5, the mid jejunum, ileum, and proximal colon all showed a greater estimate of mucin content in the THR-adequate and THR-supplemented groups than in the THR-deficient group, although none were found to be significantly different. In the duodenum, all three groups were found to be almost equal to one another.

5.3.7.4 VILLUS MORPHOLOGY-PAS/AB2.5

In the small intestine, villus height was found to be lower in the THR-deficient group than in the THR-adequate and THR-supplemented groups. In the mid jejunum and ileum, these differences were significant (**Table 5.3.7.5**). Measurements of crypt depth yielded similar trends without significant differences. Consequently, the height/depth ratio calculations yielded the same pattern of significance.

The cross sectional area at the villus-crypt junction was found to be greatest in the THR-deficient group of both the duodenum and ileum and intermediate in the mid jejunum, where these differences were not found to be significant. When the total number of goblet cells per cross sectional area was calculated, the THR-deficient group was again found to be lower than the other two treatment groups, although not significantly. Villus surface area (estimated as a cylinder with a diameter equaling the villus width at

mid villus height) was similar in trend, with the THR-deficient group lower than the other two treatment groups. Again, the differences were not found to be significant.

Villus morphology in cells stained by AB 1.0 and AB 2.5 yielded similar observations (**Table 5.3.7.6** and **Table 5.3.7.7**, respectively).

Table 5.3.7.5 Villus morphology-PAS/AB2.5

Tissue	Treatment	Height[1] (um)	Depth[2] (um)	H/D	VCA[3] (um^2)	Ttl Goblet Cells/VCA (mm)	Villus[4] S. Area ($x10^5$ um)
Duodenum	Adequate	307	205	1.56	7982	2816	1.14
	Deficient	304	177	1.80	9292	1906	1.02
	Supplem.	320	204	1.60	8325	2185	1.02
	SD_{pooled}	4.6	3.0	0.3	1253	1100	0.22
Mid Jejunum	Adequate	560^{x}	168	3.42^{a}	5581	3933	1.60
	Deficient	379^{y}	173	2.24^{b}	6096	3050	1.18
	Supplem.	403^{xy}	196	2.13^{b}	7933	4664	1.37
	SD_{pooled}	11.34	2.4	0.60	1070	2434	0.35
Ileum	Adequate	418^{xy}	185	3.89^{xy}	10571	3015	1.63
	Deficient	334^{y}	153	2.99^{y}	10621	2329	1.28
	Supplem.	516^{x}	193	5.55^{x}	7675	3857	1.69
	SD_{pooled}	9.4	4.8	1.40	1433	1095	0.30
Proximal Colon	Adequate	N/A	285	N/A	N/A	N/A	N/A
	Deficient	N/A	251	N/A	N/A	N/A	N/A
	Supplem.	N/A	294	N/A	N/A	N/A	N/A
	SD_{pooled}		50				

[1]H= villus height
[2]D= crypt depth
[3]VCA= villus/crypt cross sectional area at vb
vb= villus width at villus base (villus-crypt junction)
[4]Villus Surface Area= villus surface area at mh
mh= villus width at mid villus height

[a, b,c] denotes significance at $p < 0.05$ by Tukey's multiple comparison test
[x, y, z] denotes significance at $p < 0.05$ by LSD multiple comparison test

Table 5.3.7.6 Villus morphology-AB1.0

Tissue	Treatment	H/D (um)	VCA (um^2)	Ttl Goblet Cells/VCA (mm)	Villus S. Area ($x10^3$ um)
Duodenum	Adequate	2.32	7982	2036[a]	1.41[a]
	Deficient	1.67	9292	759[b]	1.00[b]
	Supplem.	1.84	8325	1628[ab]	1.04[b]
	SD_{pooled}	0.3	1253	543	0.20
Mid Jejunum	Adequate	3.40	5581	1369	1.58
	Deficient	2.27	6096	988	1.11
	Supplem.	2.21	7933	947	1.33
	SD_{pooled}	0.60	1070	469	0.36
Ileum	Adequate	2.46	10571	2323[ab]	1.67
	Deficient	2.56	10621	1252[b]	1.37
	Supplem.	2.38	7675	2693[a]	1.47
	SD_{pooled}	1.40	1433	760	0.20
Proximal Colon	Adequate	N/A	N/A	N/A	N/A
	Deficient	N/A	N/A	N/A	N/A
	Supplem.	N/A	N/A	N/A	N/A

[a, b,c] denotes significance at $p < 0.05$ by LSD multiple comparison test

Table 5.3.7.7 Villus morphology-AB2.5

Tissue	Treatment	H/D (um)	VCA (um^2)	Ttl Goblet Cells/VCA (mm)	Villus S. Area ($x10^5$ um)
Duodenum	Adequate	1.61	7982	2208^a	1.30^a
	Deficient	1.47	9292	594^b	0.99^b
	Supplem.	1.83	8325	1510^{ab}	0.99^b
	SD_{pooled}	0.3	1253	533	0.40
Mid Jejunum	Adequate	3.04	5581	1638^a	1.46
	Deficient	2.20	6096	1146^b	1.10
	Supplem.	3.39	7933	1093^b	1.57
	SD_{pooled}	0.60	1070	300	0.23
Ileum	Adequate	2.36	10571	2850^a	1.68
	Deficient	2.05	10621	1312^b	1.32
	Supplem.	3.41	7675	2587^{ab}	1.47
	SD_{pooled}	1.40	1433	763	0.32
Proximal Colon	Adequate	N/A	N/A	N/A	N/A
	Deficient	N/A	N/A	N/A	N/A
	Supplem.	N/A	N/A	N/A	N/A

$^{a, b,c}$ denotes significance at $p < 0.05$ by LSD multiple comparison test

5.3.8 CARBOHYDRATE ANALYSIS FOR MUCIN QUANTITATION

The amount of total mucin measured is expressed as per g of dry mucosa, per section, and as per length of gut section. The amount of mucin quantitated has been further subdivided into native (undigested by enzymes) mucin and pronase-digested mucin. The results are summarized in **Table 5.3.8.1**.

Table 5.3.8.1 Number of Native and Pronase-digested Mucins of the Gut

Tissue	Treatment	Ttl Dry Mucosa (g)	g Mucin/ g Mucosa		ug Mucin/ Section		Length (cm)	ug Mucin/ cm gut	
			Native	Pronase	Native	Pronase		Native	Pronase
Duodenum	Adequate	0.13	125.6	119.5	58.6[a]	55.8[a]	12.1	1.45	1.38
	Deficient	0.08	55.1	52.6	11.0[b]	10.5[b]	10.6	0.44	0.42
	Supplem.	0.09	114.3	108.8	49.6[ab]	47.3[ab]	11.9	1.17	1.11
	SD_{pooled}	0.05	28.8	27.3	14.3	13.5	2.0	0.37	0.35
Mid	Adequate	3.06	116.4	110.8	376.1	358.1	174.7	2.08	1.98
Jejunum	Deficient	2.28	86.4	82.8	204.4	196.2	150.6	1.44	1.39
	Supplem.	2.65	98.7	94.1	258.3	246.2	158.9	1.59	1.52
	SD_{pooled}	0.70	0.4	0.4	0.7	161	26.6	0.85	0.81
Ileum	Adequate	0.96	66.0	63.6	64.0	61.7	58.1	1.11	1.07
	Deficient	0.72	62.5	60.4	44.1	42.7	55	0.80	0.77
	Supplem.	1.00	81.9	81.1	88.3	88.5	54	1.64	1.64
	SD_{pooled}	0.29	25.6	26.9	42.7	45.4	7.7	0.75	0.80
Proximal	Adequate	0.48[a]	209.5	200.0	98.4[a]	93.9[a]	57.0	1.83[a]	1.75[a]
Colon	Deficient	0.18[b]	196.0	191.1	33.1[b]	32.2[b]	46.7	0.72[b]	0.70[b]
	Supplem.	0.31[ab]	294.0	280.7	83.7[a]	79.9[a]	55.7	1.65[a]	1.58[a]
	SD_{pooled}	0.17	76.4	73.1	37.2	35.4	9.6	0.57	0.54

[a,b] denote significance at $p < 0.05$ by LSD multiple comparison test.

In the duodenum and mid jejunum, the amount of mucin/g mucosa was found to be much higher in the adequate group than in the deficient group, although the difference

was not found to be significant due to the high variability among piglets sampled. The amount of mucin found in piglets of the supplemented group were more similar to that found in the adequate group than the deficient group. This trend was found to be similar for both native mucin and pronase-digested mucin.

In the ileum and proximal colon, the amount of native and pronase-digested mucin in the deficient group was also the lowest of the three groups but in these sections, the supplemented group contained the greatest amount of mucin. Among the different sections of gut sampled, the greatest amount of mucin was found in the proximal colon, roughly twice as much as in the duodenum and mid jejunum and more than three times as much as in the ileum.

The amount of mucin/gut section and the amount of mucin/cm gut section showed the same trends as with the amount of mucin/ g mucosa except that in the proximal colon, the differences were found to be statistically significant. Both native and pronase-digested mucin was significantly higher in the adequate and supplemented groups than in the deficient groups which were not different from each other. In the proximal colon, the amount of mucin in the adequate group was higher than in the supplemented group.

5.4 DISCUSSION

5.4.1 GROWTH OF PIGLETS

The growth of the piglets in the adequate group, measured by weight gain over the course of the study were not different from those piglets in the supplemented group. In fact, weight gain of piglets in the deficient group were also not different from the other two groups. This is most likely due to the diet being given intragastrically and not given ad libitum. Although body composition was not analyzed, it is probable that the gain in mass in the deficient piglets was due to greater fat deposition (i.e. less protein deposition) than in the other piglets. Also, the short time period of 8 days may not be adequate to show differences in growth rates in a small number of pigs.

5.4.2 NITROGEN BALANCE

Nitrogen intake, output, balance and retention values observed in the threonine-adequate and supplemented groups of the current study are in agreement with data obtained in similar studies by Chen (1997) (see also chapter 4). In the deficient group however, nitrogen output was twice as high as in the other groups, while nitrogen intake was the same. Consequently, nitrogen balance was lower, as was nitrogen retention for protein synthesis- only 70% compared to 84% in the other two groups. The lower nitrogen retention suggests that the amino acid pattern of the diet given to the deficient group was inferior to that of the other two groups in promoting protein synthesis. This is what we would expect to find because that diet is deficient in threonine. The nitrogen balance data support the plasma urea data in this respect. This also suggests that the

weight deposited in the piglets was not protein and therefore was probably fat.

5.4.3 PLASMA THREONINE

Although initial plasma threonine concentrations were similar at the onset of the experiment, prior to the introduction of test diets, they dropped almost immediately in both the adequate and deficient group while increasing in the supplemented group. While the total amount of threonine given to the piglets in the adequate and supplemented groups were the same, the different routes of administration were reflected in the final plasma concentration. As such, the lower plasma concentration of threonine in the piglets of the adequate group suggests that a large amount of orally administered threonine is retained or catabolized by the gut, in agreement with Stoll et. al (1998). The high plasma concentration in threonine supplemented piglets also suggest that threonine administered intravenously may not be as available to the gut for protein synthesis.

5.4.4 PLASMA UREA

Plasma urea concentrations may be used as an indicator of whole-body net protein synthesis. In piglets, amino acids present in relative excess are not able to be utilized for protein synthesis. Instead, they are broken down to yield ammonium ions which are then converted into urea and excreted. Plasma urea levels in the the adequate and supplemented groups remained steady and similar to each another during the study, suggesting that threonine supplied intravenously is capable of maintaining whole body protein synthesis. The plasma urea levels are similar to those found by Chen (1997) (see

also chapter 4) in similar studies. The high plasma concentration of urea in the threonine-deficient group suggests that threonine is the limiting factor in whole body protein synthesis in these piglets and the other amino acids, supplied in adequate amounts, are catabolized. This is consistent with data obtained from the previous experiment (Chapter 4) using the indicator amino acid oxidation technique.

5.4.5 DIARRHEA

A possible cause of the observed diarrhea may be bacterial or viral infection. Bacterial infections produce toxins which elicit their effects by binding to receptors on the intestinal brush border membrane, resulting in fluid secretion into the intestinal lumen, granulocyte infiltration, epithelial cell necrosis, ulceration and hemmorrhagic edema (Lylerly et al. 1988). The action of these toxins are normally inhibited by the barrier function of the mucus gel layer and alterations in its structure may inhibit its effectiveness as a barrier.

The lack of mucin may result in massive water loss. For example, the Vibrio cholerae toxin must attach itself to the villus surface in order to produce diarrhea. In order to do this, the pathogen must penetrate the mucus gel layer overlying the small intestinal mucosa (Schrank and Verwey 1976). In piglets where threonine intake is inadequate, the mucus layer is deficient in mucin proteins, resulting in a mucus gel that is lacking in carbohydate sidechains which would normally hinder the passage of toxins. The cholera toxin may then be able to penetrate the mucus barrier and elicit fluid secretion by stimulating adenylate cyclase. In rat intestinal slices incubated with crude or purified cholera toxin, both the secretion of preformed mucin and the synthesis of new mucin are

increased (Forstner et al. 1981). However, if threonine is deficient, mucins produced may not be able to perform its barrier function as well. The effect of massive fluid secretion coupled with mucus discharge would presumably to sweep away the adherent vibrios and toxins. An additional protective effect of mucin may relate to its ability to bind to toxins. It has been reported that pig gastric mucin when premixed with cholera toxin prior to instillation into rat small intestine, inhibited cholera-associated secretion. The binding of the cholera toxin to mucin was not mediated by the carbohydrate portion of the mucin but by a protein-protein interaction (Strombeck and Harrold 1974).

However, post mortems conducted in threonine deficient piglets found no evidence of bacterial infection and concluded that the deaths were due to heart failure. The diarrhea was causing dehydration, probably resulting in changes in electrolyte balance and plasma osmolarity. Diarrhea may have led to a state of hypokalemia (decreased body potassium), in turn leading to cardiac arrhythmia and acute heart failure. Therefore, the diarrhea observed in the threonine-deficient pigs may be interpreted to be a result of the the effect of the deficient treatment on mucin content in the gut.

5.4.6 TISSUE WEIGHTS

In almost all of the parameters measured, the wet lengths, weights and weight/length were all lower in the deficient group than in the adequate and supplemented groups. The lack of statistical significance may be attributed to varying water content within the samples at the time of sampling. The general direction and consistency of response in the measured parameters imply that intravenously supplied threonine does not support gut

growth and mucus production to the same extent as orally supplied threonine. With the exception of tissue weight and tissue weight/length in the small intestine, measured parameters in the deficient group were consistently lower than the other groups, suggesting that a diet deficient in threonine hinders gut growth when all other nutrients are available. It is interesting to note that the weight of the tissue/length of tissue (minus scraped mucosa) was consistently highest in the deficient group for all sections of the small intestine but not in the large intestine.

Dietary threonine most directly affects mucosal proteins and hence, the weight of mucosal scrapings. In each section of the gut examined the mucosal weight of the deficient group was lower than the supplemented group, which was lower than the adequate group. While not always statistically significant, these consistent trends suggest that threonine deficiency does not support the mucosa as well as an adequate threonine intake. Furthermore, intravenous supplementation of threonine may be able to make up some but not all of this difference.

5.4.7 HISTOCHEMISTRY

Semi-quantitative techniques of counting stained goblet cells suggest that in the small intestine, mucin production in the duodenum is most greatly affected by threonine intake. Overall mucin production, as estimated by the number of goblet cells observed, is higher in the ileum and higher still in the proximal colon. The number of goblet cells in the colon was found to be greatest in piglets receiving a deficient supply of threonine, perhaps suggesting an increased number of stem cells directed to becoming goblet cells.

Neutral mucins were found to predominate along the length of the villus while acidic mucins were found to occur mainly in the deep crypts. Regional specificity of mucins are thought to be important in maintaining the functionality of the surface mucus (chapter 2). In estimating mucin production, a simple count of goblet cells cannot be relied upon alone since some cells may be more active in mucin production than others. The inclusion of staining intensity into the equation is therefor required. This method is not without its drawbacks however. For one, staining intensity itself is a subjective measure and only semi-quantitative. Secondly, the assumption is made that all goblet cells are of the same size, which is not true. When staining intensity is taken into account, the results suggest much the same response as the goblet cell data alone; mucin production is lower in piglets fed a threonine-deficient diet than those fed a threonine-adequate diet. The inclusion of threonine either orally or intravenously seemed not to affect overall mucin production statistically although there are many occurences of the same trends which suggest that intravenous supplementation was not as effective as orally-supplied threonine in supporting mucin production.

5.4.8 VILLUS MORPHOLOGY

Villus morphology is often used as a crude measure of intestinal function. The life of an enterocyte is approximately 6 days, from formation in the crypts to being sloughed from the tips of the villi. Villus height reflects the amount of cell differentiation since intestinal cells differentiate from base to tip. Crypt depth in the intestine estimates the proliferative activities of the intestinal cells, with deeper crypts indicating a greater

number of cells present. If threonine is limiting mucosal protein synthesis, it will have an effect on villus height and/of villus height/crypt depth ratios, reducing both. In the present study, crypt depth suggested a decrease in intestinal cell proliferation in piglets fed a threonine-deficient diet. This was seen in all sections of the gut, although the differences were not statistically significant. Villus height in the mid jejunum and ileum indicated a decrease in intestinal cell differentiation in piglets fed a threonine-deficient diet, and these differences are also reflected in villus height/crypt depth ratios. Data from the duodenum exhibited similar trends, although not significantly different. In all the villus height and crypt depth data imply a similar intestinal architecture (and thus function) between piglets fed an adequate amount of threonine, either given orally or intravenously. A diet low in threonine results in altered villus architecture and thus, function.

While villus cross-sectional area yielded ambiguous results, the inclusion of the number of goblet cells resulted in trends that were comparable to other parameters of mucin production. These calculations suggest (along with villus surface area) that mucin production or at least the capacity for mucin production is depressed in piglets receiving deficient threonine in their diet. Intravenous supplementation of threonine seemed to enhance the capacity for mucin production in the distal part of the small intestine.

5.4.9 CARBOHYDRATE ANALYSIS FOR MUCIN QUANTITATION

As a more direct quantitative measure of mucin quantity in the gut, mucosal scrapings from each section were analyzed according to the alditol acetates of the carbohydrate sidechains glucosamine and galactosamine. As the diets contained only glucose as a

source of carbohydrate, any glucosamine and galactosamine measured by this process is assumed to be from endogenous production. Native, undigested mucin was estimated as a measure of total glycoslyated mucin production and pronase-digested mucin was estimated as a measure of unglycosylated mucin. In the small intestine and in the duodenum especially, differences in mucin content were not found to be significant, but this is likely due to the high variability in the samples measured. Increasing the number of animals may bear out a statistical significance. In both the small and large intestine the overall trend is of greatest mucin quantities in piglets of the threonine-adequate group, lowest in piglets of the threonine-deficient group, with those in the supplemented group being intermediate or similar to the adequate group. This suggests more directly that inadequate threonine intake does not support as much mucin production as adequate intake, and that supplementation of oral threonine by intravenous infusion is able to bridge most but not all of that gap. Also, the relative amounts of native and pronase-digested mucin were not different from one another and seems to be affected similarly by different threonine intakes.

5.5 CONCLUSIONS

Deficient threonine intake was found to result in decreased overall protein deposition in the body, as evidenced by the nitrogen balance and plasma urea analyses. Furthermore, a large portion of the reduced protein deposition was found to located in the gut, namely in the content and likely production of mucin proteins. Threonine supplied intravenously was partly available for use by the gut, with increasing towards the colon. Evidence was

found that suggests that as far as gastrointestinal mucins is concerned, the availability and/or use of orally-suppled threonine in the production of mucins was preferred. The role of threonine in the mucus lining of the gut has been demonstrated to be of utmost importance.

6. GENERAL SUMMARY AND DISCUSSION (FUTURE DIRECTIONS)

The importance of threonine in the neonatal piglet cannot be overstated and has been well demonstrated by this study. Piglets receiving intragastric nutrition require roughly twice the amount of threonine as piglets fed by intravenous infusion. The difference in requirement is likely due the the role of threonine in the piglet gut for intestinal mucins. Piglets receiving diets deficient in threonine alone show symptoms of diarrhea, increased plasma urea, and decreased mucosal weight while piglets receiving an adequate intake of threonine show no symptoms of diarrhea, and normal levels of plasma urea and mucosal weight. While these neonatal piglets are able to utilize threonine supplied either orally (intragastrically) or intravenously, there was evidence that the oral route was preferred. Given the importance of threonine in the structure and function of the gastrointestinal tract (mucus, mass), and that the rates of mucin synthesis and secretion are likely high, the dietary requirement of threonine is likely affected by changes in the gut, such as during gut atrophy and regrowth. Oral threonine during refeeding may be very beneficial to the return of 'normal' gut funcion.

A great deal of threonine reaches the large intestine in the pig in the form of mucins. Upon the degradation of mucin in the large intestine, the threonine is unlikely to be reabsorbed and is lost to the body. This would explain the higher threonine requirement in piglets fed intragastrically over those fed intravenously. In the intravenously-fed piglet, the gut is atrophied and function is greatly reduced by both need and consequence. Similarly, the amount of mucin produced and required would also be reduced, and in turn, the need for threonine.

The appearance of threonine in the large intestine upon mucin degradation may be available for use by the body, although threonine absorption in the large intestine is unlikely. Possible approaches in investigating this idea could involve the infusion of labelled threonine (such as ^{14}C-THR) into the hindgut followed by the measurement of the specific activity in the plasma. Also, the existence of specific threonine transporters in the large intestine would need to be investigated. Another experiment would entail the labelling of threonine in feed, followed by the measurement of labelled threonine in the feces.

The role of gut hormones cannot be ignored, especially during times of gastrointestinal stress. Hormones that affect intestinal motility (secretin, motilin, neurotensin, peptide YY) may act and interact during threonine deficiency. There is also some evidence that enteroglucagon could be a physiologic regulator of cell turnover in the gut and somatostatin has been shown to inhibit amino acid absorption, ileal contraction tissue growth and splanchnic blood flow. Clearly, any dietary effect of threonine on mucin production and content would include responses from these hormones.

The possibility of mucin gene upregulation during times of prolonged dietary deficiency could be investigated by use of cell cultures. Specific intestinal mucin genes isolated and cloned such as MUC2 (in LS174T human colon carcinoma cells and LS180 colonic tumour cells) and MUC3. MUC2 is the prominent mucin in human large intestine, specifically in goblet cells while MUC3 is largely confined to enterocytes. The direct effects of threonine could thus be investigated, via the use of MUC2 and MUC3 antibodies in immunoprecipitation studies.

As threonine is important in maintaining the mucus layer, it would be of interest to investigate the effects of threonine during refeeding of intravenously-fed subjects. Perhaps a threonine enriched oral diet during refeeding or even an enriched parenteral diet may speed up gastrointestinal recovery in post surgical subjects.

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